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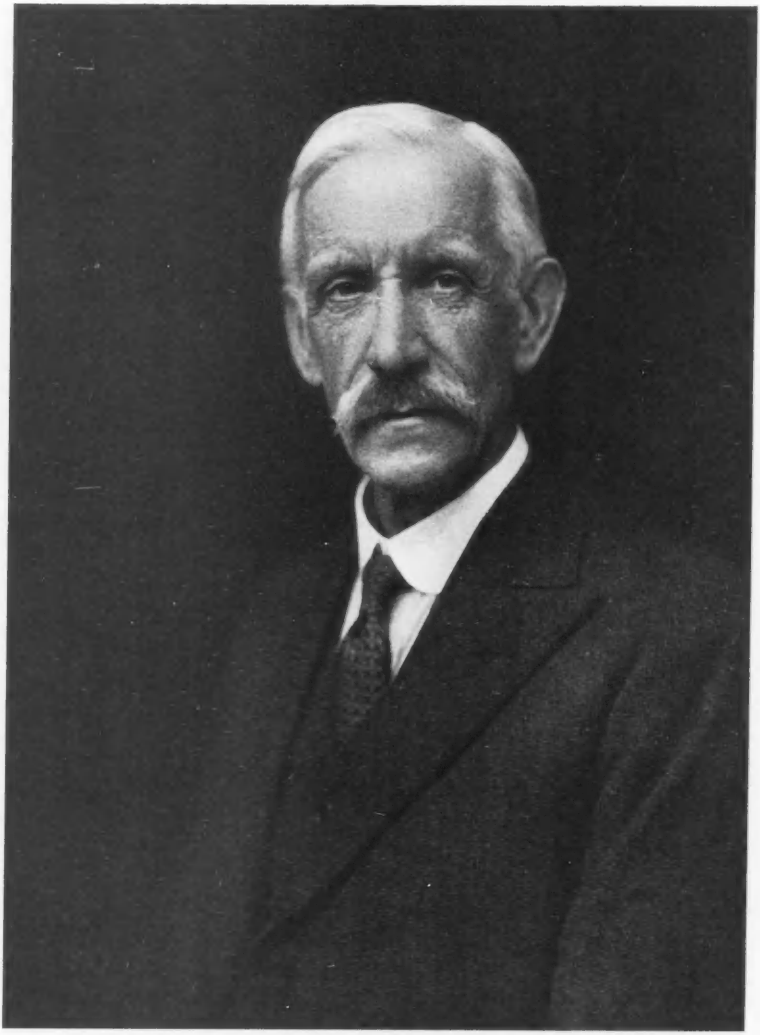

FREDERICK GOWLAND HOPKINS

Obituary Notice

FREDERICK GOWLAND HOPKINS, 1861–1947

PART 1. LIFE

Early life

Frederick Gowland Hopkins was born in Eastbourne on 20 June 1861. His father was not a professional scientist but had definite scientific leanings, being at one time associated with his second cousin, Sir Frederick Abel, in the latter's work on tides and currents. Unfortunately his father died while he was an infant and the rearing and educational guidance of the child devolved on the widow, who was assisted and guided by her brother, James Gowland.

From the age of six to ten Frederick Hopkins attended a dame school in Eastbourne; being the only day boy, he led a rather lonely life, his greatest recorded pleasure being in the use of his father's microscope about which he wrote:

> I felt in my bones that the powers of the microscope thus revealed to me were something very *important*—the most important thing I had yet come up against; so much more significant than anything I was being taught at school.

In 1871 the mother and son moved to Enfield to live with the uncle aforementioned and Hopkins's maternal grandmother. The boy was then sent to the City of London School, situated at that time in its old dismal surroundings in Honey Lane. This school seems to have done little to cater for the tastes and intelligence of the boy and he left at the age of fourteen, after several weeks of truancy, innocently occupied in country walks and in visits to museums and public libraries. This escapade, which he attributed to boredom, led to his removal to a private school where the educational advantages were of a low order. He left school for good at the age of seventeen, and, under pressure from his uncle, entered a firm of insurance agents. This unfortunate choice was not dictated by straitened means, but appears to have been made by a well-meaning but unimaginative relative as likely to provide a safe and satisfactory career. Hopkins stayed in this prison for six months only—long enough to learn the art of adding the three £ s. d. columns simultaneously—and escaped owing to the intervention of a family friend, who discerned in the boy an aptitude for science and advised his elders accordingly. Even now Hopkins was not sent to a university, though his father's friend and cousin, Sir Frederick Abel, whose advice was asked, said 'Cambridge'. This was, however, considered likely to provide too theoretical a training and Hopkins became an articled pupil in a private analytical laboratory run for profit. Here he spent the next three years in analytical practice, and records that, apart from manipulative skill, he learnt nothing worth while.

At the age of twenty one Hopkins became possessed of a small legacy from his paternal grandfather, and decided on his own initiative to embark on a scientific training at the Royal School of Mines in South Kensington. He was soon invited to become an assistant in the private laboratory of Dr Percy Faraday Frankland, where analytical work on railway material was in progress; however, at the end of six months this concern closed down and Hopkins was again at a loose end. This time he decided to work for the Fellowship of the Institute of Chemistry, and attended classes at University College, London, for that purpose. He records that he did exceptionally well in the examination and, as a result, was invited by Dr (afterwards Sir Thomas) Stevenson, the Home Office analyst and medical jurist, to become his assistant in his laboratory, then located in Guy's Hospital. Hopkins has stated that the five years he spent here were very happy ones; he learnt much, made lasting friends and worked on many famous and interesting cases. During his second year with Stevenson he and his colleague, Wade, decided to work for the London external degree; they both matriculated in 1887, entirely as the result of studies carried out in the evenings and on their daily railway journeys.

So far Hopkins's training had been purely chemical, but his position in Stevenson's department in Guy's Hospital brought him in contact with medicine and medical students. One of these (Sir) Charles Martin, recalls how he was wont to cultivate his taste for chemistry in the laboratory where Hopkins worked and to chat with the young analyst on problems of physiological chemistry which already fascinated both of them; it seems indeed to have been Martin himself who first suggested to Hopkins the idea of abandoning analytical chemistry and taking up research on physiological chemistry, and the urge to take this step evidently became stronger and stronger. In those days the main pathway into this field led through the study of medicine, and this consideration finally led Hopkins to interrupt his work as a chemist in order to undertake the arduous task of a medical training. In 1888, at the age of twenty seven he entered Guy's Hospital as a medical student, qualifying in science and medicine

simultaneously. He passed the Intermediate B.Sc. in 1888 and B.Sc. in 1890 (honours in Chemistry and Physiology and gold medal in Chemistry). In the same year he passed the Intermediate M.B. and in 1894 the M.R.C.S. and L.R.C.P. and the London M.B.; his D.Sc. followed in 1900. During this period he held a studentship founded in memory of Sir William Gull.

Whilst still a student, and during the following years, he carried out research on a variety of subjects mainly arising from clinical studies and, after qualifying, soon established himself as a consulting clinician. He was also one of those who started and worked for the 'Clinical Research Association', an enterprise which undertook to examine and report on pathological, bacteriological and biochemical material sent in by medical practitioners—work now undertaken by public health laboratories but then uncatered for. Life at this time was very arduous but prospects were bright.

In 1898 Hopkins married Miss Jessie Stevens, daughter of Edward Stevens of St Lawrence, Kent. This event was of more importance to biochemistry than is often recognized. Had Hopkins added to the claims of a widowed mother (which he always paid in full measure) the exactions of a wife who was unable to appreciate his potential position as a scientist, or one who was apt to consider her own claims as important, the course of his career might well have been in clinical medicine, where he was already making both a name and an income, rather than in scientific research. Within a few months of their marriage, while they were living in Lincoln's Inn, the couple were faced with the parting ways. At the meeting of the Physiological Society held in Cambridge in 1898 Sir Michael Foster invited Hopkins to come to the Cambridge Physiological Laboratory, where he himself held the chair, to develop teaching and research in physiological chemistry. It was a difficult decision; all Hopkins's scientific ambitions and tastes urged him to accept the invitation, but common prudence insisted that he was already in a very favourable professional and financial position in clinical medicine, and leaving all this to go to Cambridge on a small income involved burning a veritable fleet of boats. Mrs Hopkins, herself connected with medicine through her profession as a nurse, might well have been expected to emphasize the latter consideration, but fortunately for science all her weight was on the other side, and it turned the scale; the Hopkins couple came to Cambridge in the same year. Throughout their lives Mrs (now Lady) Hopkins never wavered in supporting her husband in following, at whatever financial loss or risk, his career in research rather than anything else. Her own relations with his department were always smooth and easy; she was everyone's friend and nobody's enemy. Biochemists owe her more than they are ever likely to know.

The early Cambridge period

It need not be supposed that life in Cambridge was a change in all respects for the better. As usual in those days, his University stipend was quite inadequate for even the most modest requirements of a married couple. It was supplemented by Emmanuel College which appointed him supervisor of medical students and later (1906) Tutor in Science; this involved personal teaching both in physiology and anatomy; the latter was a real grind and in later years the Professor never alluded to it without making a wry face, and Lady Hopkins recalls how she was wont to sit up till 2 a.m. for fear her husband should neglect to make up the fire while he literally swotted up this uncongenial subject, which he had not touched since his student days.

These adverse circumstances remained unmitigated longer than might have been expected owing to the untimely death, in 1907, of Sir Michael Foster, who had been responsible for bringing Hopkins to Cambridge and was deeply interested in the success of the enterprise. But in spite of some attendant austerities things soon began to move along. The development of research during this period is peculiarly interesting but belongs to the later section of this article, but it must be recorded here that his University position was recognized and stabilized by the creation, in 1902, of a Readership in Physiological Chemistry, which at any rate implied that in his four years at Cambridge, Hopkins had convinced some authorities that the subject existed and was worthy of support. In 1905 he was elected to a Fellowship of the Royal Society. In 1910 his circumstances were eased by his election to a Fellowship in Trinity College and a Praelectorship in Physiological Chemistry. This enabled him to give up his arduous teaching in Emmanuel, which notified its appreciation of his value by electing him to an honorary Fellowship in the following year. Hopkins always recalled with gratitude his connexion with Emmanuel and the help it afforded him in his early years in Cambridge, and his elder daughter was married in the College Chapel. The connexion with Trinity, begun at this time, was also a great source of pleasure and satisfaction, a feeling which was surely fully reciprocated by those Fellows who enjoyed the wisdom of his counsels and the pleasure of his society. Hopkins speaks of his appointment to the Praelectorship thus:

> ...for me the election was salvation and my debt to Trinity is great indeed. For 26 years I have received all the great privileges that membership of the College confers and have been asked for no single service in return. I know well that my election was in the first place due to the

generous advocacy of Walter Fletcher. It is my hope that in any account of my career published after my departure the generosity of Trinity College will be emphasized.

Second Cambridge period. Recognition and growing success

In 1914 a special Chair of Biochemistry was created for Hopkins, but he did not at this time get a new building as was at first hoped; instead this went to Physiology whilst Hopkins, as representing the junior subject, had to content himself with the old building in Corn Exchange Street vacated by the senior science. Nevertheless he now had a chair, a professorial income and a department. He had established Biochemistry in Cambridge, indeed in England.

In the same year came the first world war, and Hopkins turned over his laboratory to the study of war problems, mainly nutritional. He became one of the original members of the Medical Research Committee (later Council) whose first Secretary was his friend and collaborator, Walter Fletcher; he worked on numerous committees, advising the Government on food policy and medical matters. By the end of the war Hopkins's scientific value was recognized far and wide.

In 1920, through the generosity of the Beit Trustees, he was able to expand his Department by the acquisition of the Balfour laboratory into which his rapidly increasing number of research workers was able to overflow.

Later Cambridge period. Achievement and honours

In 1921 part of the bequest of Sir William Dunn was devoted by the trustees to the building of a laboratory and the endowment of a Chair of Biochemistry in the University, and the subject was thus permanently established in Cambridge; the new laboratory was occupied in 1924. Honours and recognition now flowed thick and fast and it is impossible to enumerate them all. In 1921 he was invited to deliver the Herter lectures at Johns Hopkins University, and he and his wife made their first visit to the States, cementing earlier friendships and making many new ones. He was knighted in 1925, received the Copley medal of the Royal Society in 1926, and in the same year delivered the inaugural address to the International Physiological Congress in Stockholm. In 1929 he shared with Eijkman the Nobel Prize for Medicine. He was elected President of the Royal Society in 1931 and appointed to the Order of Merit in 1935.

In 1939 he underwent an operation after which his health woefully declined; he became increasingly feeble in body (though never in mind) and nearly blind. He carried on laboratory work, with the help of his assistant, James Morgan, till the end and his last seven papers were the result of work done in these difficult circumstances.

He died on 16 May 1947. His wife, Lady Hopkins, who contributed so much to all aspects of his life and cared for him with such skill and devotion during his last years, survives him. He leaves a son in the medical profession and two daughters, Barbara Holmes and Jacquetta Hawkes, distinguished in biochemistry and archaeology respectively.

PART 2. SCIENTIFIC WORK

It is convenient to divide Hopkins's scientific work into the same chronological periods as were used in the outline of his life.

Early life

As a boy Frederick Hopkins was interested in butterflies and beetles and his first published paper, written at the age of seventeen, was a letter to the *Entomologist* on the habits of *Brachinus crepitans* (Hopkins, 1878). His early entomological studies must have influenced his thoughts considerably for, apart from this letter, three out of his first six published papers were studies on the pigments of the *Pieridae*. This work culminated in a paper published in the *Philosophical Transactions of the Royal Society* (Hopkins, 1896) in which the white pigment of the common cabbage butterfly was identified with uric acid, and the fact that an excretory product finally turned up as an ornamental pigment emphasized. Actually the identification of the main pigment with uric acid was incorrect, a mistake which he recognized when he returned to the subject very late in his life (Hopkins, 1942), but its close chemical relation with uric acid and its appearance as an excretory product together with lepidoporphyrin, the yellow pigment of the related brimstone butterfly, in the rectum of the pupa shortly before the emergence of the butterfly struck him very forcibly. The work as a whole unites the skill of the analyst with the knowledge and outlook of the biologist, and already denotes the dual nature of the writer's scientific character.

Problems in uric acid excretion occupied a good deal of attention during this period, probably owing to the influence of Garrod. A reliable method was perfected for estimating uric acid in urine (Hopkins, 1893) and used in a study of uric acid metabolism (Hopkins & Hope, 1898–9). It was here shown for the first time that the nucleins of the diet were not alone responsible for the peak rise in uric acid and excretion after food.

A fine study on urobilin was done in conjunction with Garrod in this period (Garrod & Hopkins, 1896) in which Hopkins obviously contributed the method of separating urobilin in a purified state from normal

and pathological urines, faeces and bile, and both contributors showed by the use of the spectroscope the identity of the products from the four sources; this paper is characteristic of Hopkins's exact description of the methods employed in the separation, giving at each stage the reasons for the precautions used and taking obvious pleasure in the technical details.

Hopkins began his protein work at this time with studies of the compounds formed by these substances with halogens (Hopkins & Brooks, 1897–8; Hopkins & Pinkus, 1898–9) and in preliminary accounts of the crystallization of egg albumin (Hopkins, 1898*a*). A comprehensive article on urine written for Schäfer's text-book also belongs to this period (Hopkins, 1898*b*).

Early Cambridge period

The first paper to be published after moving to Cambridge was the still famous description of the crystallization of egg albumin, the preliminary studies for which had been begun in London. Hopkins's interest in proteins had many facets; first, as the principal constituent of 'protoplasm' they demanded attention from anyone claiming to attack the chemistry of the living organism; secondly, so long as the chemistry of proteins remained vague, support was lent to those who claimed that the study of the chemistry of life was a hopeless endeavour. Hopkins doubtless felt that to obtain proteins in a crystalline form was a step towards reconciling chemists to undertake their study, though he himself entertained no illusions as to the significance of the crystalline state with respect to these substances. Having, however, replaced the crude procedure of Hofmeister by carrying out the precipitation of the protein by ammonium sulphate at a controlled pH using dilute acetic acid, he transformed a difficult and uncertain operation into one of ease and certainty, capable of giving high yields; this same method is in frequent use to-day in the purification of enzyme proteins. By carrying out measurements on the optical rotation and estimations of C, H, N and S on samples which had been subjected to a varying number of recrystallizations, Hopkins showed that he was dealing with a chemical entity; at that period this was a great advance in the study of proteins, and helped to eradicate the view that they were unstable entities of fluctuating composition. Having achieved this, Hopkins did not repeat the performance with other proteins, nor did he set his pupils to gain easy credit by crystallizing a series of these compounds as many a lesser man would have been tempted to do; instead he turned to other problems.

In the next three papers, also concerned with proteins, Hopkins had the collaboration of S. W. Cole who was then beginning his career in research and teaching. These form a logical series of great importance and interest, the stimulus for which arose from an amusing accident. It was customary in those days (and still is) for elementary students to be introduced to the study of proteins by performing a series of colour reactions. On one occasion the entire class failed to perform the Adamkiewicz reaction—then completely empirical—consisting in the production of a violet colour on the addition of dilute acetic followed by strong sulphuric acid. This led to the discovery that if the acetic acid was pure the test was always negative, but that the active agent could be separated from samples giving the test either by distillation or by crystallizing out the acetic acid. After an amusing and highly informative piece of research, the active agent was proved beyond doubt, by a number of rigorous tests, to be glyoxylic acid and the test thereafter became known as the glyoxylic test (Hopkins & Cole, 1901*a*). Having succeeded in identifying the active participant in the reagent, the same authors were moved to search for the group or unit in the protein molecule with which it reacted. This led to the famous isolation of tryptophan (Hopkins & Cole, 1901*b*). This work is too well known to need comment, but it may be noted that here Hopkins used for the first time his favourite precipitating agent, viz. 10% mercuric sulphate in 5% sulphuric acid.

The same authors (1903) sought to establish the identity of the new amino-acid by a highly original and biological method, viz. the identification of various products of its bacterial decomposition. Using as a substrate 2 g. tryptophan in 2% gelatin they obtained by the action of *Esch. coli*, aerobically, both indole and indolylacetic acid and by *Cl. chauvei* and *Esch. coli* anaerobically indolylpropionic acid. Unfortunately the three last compounds were, on the basis of early work, erroneously identified as their skatole isomers, i.e. skatolyl-2-carboxylic instead of β-indolylacetic and skatolyl-2-acetic in place of β-indolyl-propionic acids; this led to the formula first assigned to tryptophan being skatolyl-2-amino-acetic acid, a mistake which was only cleared up when tryptophan was synthesized from β-indolyl-aldehyde and hippuric acid (Ellinger & Flamand, 1907).

Hopkins's biological interests led him immediately to investigate the importance of tryptophan in mammalian diet (Willcock & Hopkins, 1907). Zein was used as the protein, this being known to be deficient in both tryptophan and lysine; young (and later adult) mice were the experimental animals. The results were without ambiguity:

> Zein, it is clear, has no power whatever of maintaining growth in the young animal, loss of weight begins the moment it forms the sole nitrogenous supply. The addition of the missing tryptophan group to the zein has, it is also clear, no power to convert such loss into equilibrium or

gain; a fact possibly due to other deficiencies in the zein molecule, such as the absence of lysine, or the lack of some other amino-acid not yet observed.

On the other hand the benefit of added tryptophan was clear; it almost doubled the survival period and eliminated the marked symptoms of lethargy noted on the unsupplemented zein. This study is important as introducing the knowledge that certain amino-acids are essential to the mammalian organism and, when absent from the protein food, can be supplied as such in the dietary.

Soon after this important work came the joint study with Fletcher on lactic acid in muscle (Fletcher & Hopkins, 1907). Fletcher had been working on this subject since 1898 and had already begun the study of CO_2 production in muscle on rational lines; when Hopkins joined in the work soon after coming to Cambridge a very fruitful collaboration began. The paper of 1907 is important for several reasons. By the use of ice-cold ethanol, lactic acid was extracted without stimulating the muscle and an important source of error avoided. Thus it was possible to demonstrate the accumulation of lactic acid in fatigue during anaerobiosis, and its removal in oxygen, and also the fact that the production of lactic acid in heat rigor is the same in resting muscle as in muscle which has been stimulated for 50 hr., whilst producing lactic acid over the whole of that period.

The great importance of this work is that it started the study of muscle fermentation and its relation to muscular contraction, a study which in the years that followed attracted many notable workers from all parts of the world, and developed into one of the most complete achievements of modern biochemistry providing, even to-day, material for work of major interest and importance. It also stimulated important work on biological oxidations.

Between 1906 and 1911 Hopkins published no major work, but there is evidence that he was employed in nutritional experiments which remained unpublished. In 1911 came a fine study on the Bence-Jones protein (Hopkins & Savory, 1911); this comprised a complete examination of the amino-acids of the protein by the methods of Kossel and of Fisher in addition to some originated by himself. Physical and analytical studies were also made, the whole illustrating Hopkins's wide command of technique. To all this were added important metabolic studies on the relation of the Bence-Jones protein in the nitrogen metabolism of the patient.

In 1912 was published what was perhaps the most famous of Hopkins's contributions to science, entitled 'Feeding experiments illustrating the importance of accessory factors in normal dietaries' (Hopkins, 1912). This work, as is invariably the case with communications of major importance, did not appear unheralded. The idea of deficiency diseases had been in the air for some time; Eijkman in 1890 and Grijns in 1901 had established the fact that beriberi was a deficiency disease due to a diet of over-milled rice; and in 1914 Funk was attempting the isolation of the active substance from rice polishings. Scurvy was believed to be due to a dietary deficiency owing to the work of Holst and Frölich. More historically startling is the fact that already in 1888 Lunin, working in Bunge's laboratory in Basle, had found that mice fed on the proteins, fats, carbohydrates and salts of milk failed to survive and concluded that milk must contain unknown substances necessary to life. Later Socin (1891), working in the same laboratory, showed that milk and eggs in small quantities rendered the synthetic diet adequate for growth and maintenance. Both these papers remained quite unnoticed, and in his Nobel Lecture of 11 December 1929 Hopkins discusses this misadventure at some length. Much later, in 1905, Pekelharing pointed out that there existed in milk a substance which, when present only in very small amounts, is of paramount importance in nutrition. Even at this late date Pekelharing's work attracted no attention, probably because it was written in Dutch and failed to get into the abstracts.

For many years Hopkins had had in mind the possibility that something more than pure proteins, fats, carbohydrates and salts might be necessary for growth and even for maintenance. He used to relate how, when a student at Guy's Hospital, two sailors were brought in with scurvy, and lay in adjacent beds while subject to the citric acid treatment used at that time. One day a relative of one of them brought him a bag of oranges; the subsequent spectacular recovery of the fortunate recipient did not escape the eye of the medical student, and seeds of doubt on accepted principles of diet were then sown to bear fruit later. Already in 1906 Hopkins in addressing the Society of Public Analysts committed himself to the following statement:

> No animal can live upon a mixture of pure protein, fat and carbohydrate, and even when the necessary inorganic material is carefully supplied the animal still cannot flourish.

In his Nobel Prize lecture (Hopkins, 1930) he admits to delaying the results of his earlier experiments owing to the

> great temptation to endeavour to isolate the active substance or substances before publication and I can claim that throughout the year 1909 I was engaged upon such attempts though without success.

Considering the complexity of the water soluble B group of vitamins this failure is not surprising. In his famous paper of 1912 Hopkins showed that a dietary consisting of casein, extracted exhaustively with ethanol, starch, sugar, lard and inorganic salts

was unable to maintain growth or support life in young rats, but became perfectly adequate when fresh milk amounting to 1–3 % of the whole diet was added. The great feature of the work was the complete adequacy of the controls introduced in the selection of the animals, and the certainty that incomplete ingestion or absorption were playing no part. As a perfect experiment of its kind it stands as a model.

Second Cambridge period

In 1916 Hopkins returned to the study of the effects of individual amino-acids in the diet (Ackroyd & Hopkins, 1916), and made the important discovery that either arginine or histidine were necessary, the one being replaceable by the other; their role as precursors of allantoin was also shown. Throughout the period of the first world war, much of Hopkins's time and energy was occupied in popularizing the conception of the complexity of dietetic requirements, and the necessity for the supply of special molecular configurations, whether as vitamins or amino-acids, in opposition to the older notion that calories and proteins alone were the prime considerations in compiling an adequate diet.

Later Cambridge period

Towards the end of the first world war, Hopkins had become interested, through some experiments on the sulphur compounds of the cell, in a substance recognized as containing an SH group, found to have a very wide distribution in growing cells of both plants and animals. In 1921 he succeeded in isolating the substance from yeast and published a paper entitled 'An autoxidizable constituent of the cell' (Hopkins, 1921*a*). Hopkins called his substance glutathione, at that time believing it to be a dipeptide of glutamic acid and cysteine. This discovery inaugurated a long series of studies, both by Hopkins himself and by numerous pupils, on cellular oxidations in which this important peptide played the part of catalyst, and certainly orientated the activities of his department in the direction of biological oxidations for many years. In 1927 the constitution of glutathione was challenged by Hunter & Eagles, and Hopkins set about a re-examination of its structure (Hopkins, 1929). In the course of this work he completely changed the method of isolation, using a new and very beautiful technique which consisted in the precipitation of the peptide as a cuprous salt by the addition of cuprous oxide in the presence of 0·5N-H_2SO_4. The peptide came down in a high state of purity and proved to be a tripeptide of glutamic acid, glycine and cysteine. This discovery put the study of glutathione on a new and sounder footing; it also provided an example of a new and very elegant method for the isolation of the substance.

In 1921 Hopkins crystallized his views on biological oxidations, including the functions of glutathione, and on the position of muscle chemistry in the two Herter lectures which he delivered before the Johns Hopkins University in the spring of that year; on the same visit to the States he received the Chandler medal and devoted the address delivered on that occasion to the nutrition problem (Hopkins, 1921*b*, *c*; 1922). The lectures familiarized American biochemists with Hopkins's individual and original approach to the subject and resulted in a considerable flow of American workers to his laboratory in the succeeding years.

In 1923 he returned to his purine studies and, in collaboration with Morgan & Stewart, published the classical paper on the oxidation of xanthine and hypoxanthine to uric acid by the enzyme xanthine oxidase found in milk and animal tissues (Morgan, Stewart & Hopkins, 1922).

From 1931 to 1936 Hopkins was so much occupied with his duties as President of the Royal Society that his personal experimental work was sadly interrupted, and his published work during this period consisted largely of lectures and addresses. He returned to his laboratory in 1937, and then worked largely on problems connected with the function of thiol groups in enzymes, and drew attention to the part they play in maintaining active substances in the reduced condition in the cell.

In 1939 his period of bad health and failing eyesight set in, and he was hampered in his choice of subjects of research for this reason. In 1942 he returned to the study of butterfly pigments, and published an interesting paper on the pterins and their oxidation products (Hopkins, 1942). In his last years many of his earlier studies were resumed. Glutathione appears in two papers; in the first (Hopkins & Morgan, 1943) it was shown that, in many seeds, glutathione appears and increases in concentration in the early stages of germination. From 1·1 kg. of germinating pea seeds it was found possible to isolate sufficient glutathione in the crystalline state to serve for identification.

The second paper concerned the distribution of glyoxalase and its specific coenzyme, glutathione, in a large number of plants and invertebrate animals. Both the enzyme and coenzyme were found in almost every plant and animal tissue examined (Hopkins & Morgan, 1945). It is clear from the paper, and was emphasized in private conversation, that the discovery of the wide distribution of this enzyme throughout many divergent groups of organisms, whilst its natural substrate remained uncertain, was a source of worry and perplexity to Hopkins, and he earnestly recommended the problem to the notice of research workers. The fact that a function of glutathione is so closely involved in the problem doubtless increased its fascination for him.

In 1944 appeared a study of hypoxanthine, recalling Hopkins's interest in purine metabolism. This work was done in collaboration with Mrs Simon-Reuss who brought her experience of tissue culture to bear on the problem. Here it was shown that the addition of hypoxanthine in a concentration of 15 μg./ml. to the tissue culture media very markedly increased both the rate and duration of the tissue growth whilst adenine had no such effect, suggesting that the hypoxanthine supplement enabled the cells to synthesize nucleins more readily (Hopkins & Simon-Reuss, 1944). In 1945 Hopkins published work in which he examined the possibility that refection might have been in part responsible for his results in the vitamin work of 1912 (Hopkins & Leader, 1945). Here, using a technique due to Miss Leader, the phenomenon of refection was completely eliminated by the administration of filter paper as roughage. Rats so fed never showed refection owing, apparently, to the rapid removal of the undigested starch from the gut and the consequent prevention of excessive proliferation of bacteria and yeasts in the caecum. With animals so treated the effect of 3 ml. of milk/rat/day was as effective in maintaining growth as in the experiments of 1912, whilst the decline in the absence of the milk was equally unequivocal. In this study the reason for the association of refection with the use of potato starch as the main carbohydrate source was carefully studied, but no satisfactory solution to this problem was found.

A study on glyoxalase (Hopkins & Morgan, 1947) was in the press when Sir Frederick Hopkins died.

PART 3. TEACHING AND SCIENTIFIC INFLUENCE

A man's scientific achievement is the combined result of his scientific ability and his character. In no one is this more clearly exemplified than in Sir Frederick Hopkins. On looking back over the years it seems that he was destined from the first to occupy a unique position in science. He united an aptitude for chemistry, brought out by his training as an analyst, with an intense appreciation of biology; this is especially apparent in his early papers. To these qualifications he added a consciousness of having a mission with respect to the development of biochemistry, and a scientific insight amounting to genius. When he entered the field, biochemistry in England lagged far behind the developments on the continent. There, such workers as Kossel, Salkowski, Hoppe-Seyler and Emil Fischer had produced brilliant studies on compounds of importance in the animal and plant; no one of the calibre of these men had worked on similar problems in this country. But, conscious as Hopkins was of the high importance and value of these achievements in the structural chemistry of what may be termed the fundamental organic chemistry of the animal, it was not in this direction alone that he conceived biochemistry should advance. What fascinated him was not the static chemistry of the organic chemist, but the problem of the chemical changes which occur within the animal and plant cell and their relation to function. But the possibility of such studies appealed to very few persons capable of making them, and Hopkins set himself by achievement and by propaganda to induce a change of thought among chemists of his day. In an address to Public Analysts in 1906 he said:

> The scientific chemist—unlike his predecessors the pioneers of sixty or seventy years ago—has long since ceased to be much interested in the animal or the plant. Further, the triumph of synthetic work in advancing theory has led the pure chemist away from the special difficulties of analytical work. His extraordinarily developed technique concerns itself only secondarily and imperfectly with analytical studies of the kind still necessary in physiological problems, I mean the endeavours to identify and separate unknown substances with unknown properties present in complex mixtures. Such work requires special instincts and the pure chemist has largely lost them. He is but a poor analyst as the physiological explorer finds on turning to him for help (Hopkins, 1906).

Hopkins made also a bold plea for co-operation on equal footing of the chemist and the medical man:

> The care of the body in sickness must remain always an entire and carefully guarded prerogative of the physician; but the innate respect of the public...for the physician's calling has led to a somewhat illogical attitude and has tended to make sacrosanct not only the calling of the physician but the scientific material which he deals with.... The progress of scientific medicine demands a change here. While a large part of future scientific medical studies must always be carried out by men who, though medically qualified, have preferred the laboratory to practice...yet in a middle region these must be joined in their work by men whose primary qualifications are non-medical, men who, saved from the long years of clinical study, are able to bring well grounded laboratory knowledge and (I may add) a sufficient knowledge of the literature of pathology...to join their medically qualified confreres in attacking the huge problems which await solution (Hopkins, 1906).

Hopkins also felt the need to modify the attitude of physiologists towards biochemical studies; again his attitude is expressed best in his own words:

> I wish, however, to remind you of a mode of thought concerning the material basis of life which...was almost as inhibitory to productive chemical thought and study as any of the claims of vitalism. This was the conception of that material basis as a single entity as a definite though highly complex chemical compound. Up to the end of last century and even later, the term 'protoplasm' suggested such an entity to many minds.

To exemplify this he quotes Sir Michael Foster, whom he nevertheless revered, as saying,

> The oxygen which enters the muscle from the blood is not involved in immediate oxidations, but is built into the substance of the muscle. It disappears into some protoplasmic complex... we cannot yet trace the steps taken by the oxygen from the moment it slips from the blood into the muscle substance to the moment when it issues united with the carbon as carbonic acid. The whole mystery of life lies hidden in that process, and for the present we must be content with knowing the beginning and the end.

On which Hopkins comments:

> So long as the term 'protoplasm' retains a morphological significance as in classical cytology it may be even now convenient enough though always denoting an abstraction. Insofar, however, as the progress of metabolism... was ascribed in concrete thought to hypothetical qualities emergent from a protoplasmic complex in its integrity or when substances were held to suffer change only because in each living cell they are first built up, with loss of their own molecular structure and identity, into this complex, which is itself the inscrutable seat of cyclic change, then serious obscurantism was involved (Hopkins, 1935).

Hopkins disliked the conception of what he used to term the 'giant molecule' of living matter, within which mysterious chemical or pseudo-chemical events were supposed to occur, because he believed that it was inhibitory to biochemical thought and progress. So long as it remained, biochemistry, as he conceived it, would never develop because no one would attempt to solve the problems of chemical change within the body unless they were conceived of as ordinary chemical problems. Again and again Hopkins, in lectures, addresses and private conversations, returned to flog the 'giant molecule', just as Pasteur returned again and again to attack the heresy of 'spontaneous generation'. Until these ideas were removed from men's minds, Pasteur and Hopkins alike felt convinced that their respective sciences could not progress.

Hopkins early became convinced of the importance of chemical specificity in the organic molecules utilized by the animal. In a remarkable review paper in *Science Progress* 1906–7 he deals with the utilization of proteins by the animal, starting from the views of Liebig, to whom, as he humorously remarks, 'the fundamental aspects of protein metabolism in the animal appear to suggest no great difficulties' and 'whose answers to the questions partake of the lusty self confidence characteristic of biological science of his day'. He then proceeded to outline the evidence of recent work on the absorption from the gut, not of proteins, but of the constituent amino-acids, and outlined all the evidence extant at the time on protein digestion and utilization. In considering the 'remoulding' of the amino-acids into the proteins appropriate to the different organs of different animals he made the following prophetic utterance:

> Evidence is accumulating to show that there is a chemistry of species; that chemical differences in the protoplasm underlie morphological differences and that these differences concern in particular the complex and easily variable proteids.

The evidence accumulating with the years confirmed Hopkins in his conviction of the importance of specificity and by 1935 he was prepared to replace biogen or the giant molecule by a system in accord with modern research:

> Were it not equipped with catalysts, every living unit would be a static system.
>
> ...Catalysis is one of the most significant devices of nature, since it has endowed living systems with their fundamental character as transformers of energy, and all evidence suggests that it must have played an indispensable part in the living universe from the earliest stages of evolution.
>
> Life is manifested in chemical systems which it seems must have remained inert but for its influence. One would dearly like to know whether life at its origin was displayed by systems which could dispense with it, and if not what was the nature of the primitive catalyst.
>
> A molecule within the system of the cell may remain in an inactive state and enter into no reactions until at one... surface it comes in contact with an enzymic structure which displays certain adjustments to its own structure.
>
> An enzyme is in general adjusted to come into effective relations with one kind of molecule only or at most with molecules closely related in their structure.... The one aspect of enzymic catalysis which for the sake of my theme I wish to emphasize is its high specificity (Hopkins, 1935).

Hopkins's own character contributed greatly to his success in persuading scientists to consider and ultimately to accept his views. He seldom antagonized opinion and never quarrelled; his relationship with his juniors was always stimulating and never authoritative. He could not tolerate strained relationship or personal quarrels and his department was singularly free from them. Nevertheless, he inhabited no ivory tower. Throughout his life he laboured unceasingly to establish biochemistry as a 'separate discipline' and, so far as Cambridge University was concerned, he succeeded. The early course of recognition of biochemistry as a subject is marked by the steps in the recognition of Hopkins in the University. As soon as he became master of an adequate laboratory (1924) he established Biochemistry as a subject for Part II of the Tripos and the formal teaching of advanced students began. In this course all senior members of the Department contributed their quota and young biochemists began to flow from Cambridge into scientific activities of various kinds. In 1938 Biochemistry was established as a subject for Part I of the tripos, giving to every science student in Cambridge the

opportunity of acquiring an elementary knowledge of the subject should he so desire.

It need not be supposed that Hopkins achieved all this by lying on a bed of roses. He was personally beloved, but in establishing biochemistry he met with considerable opposition and criticism from the exponents of subjects upon the confines of which it bordered, and from non-scientists. But when the time came he never shrank from applying the necessary pressure and his many friends stood by ready to help. It is, moreover, noteworthy that at the time of his death only one other University in England, and one in Scotland, had established biochemistry as a subject for a degree, or attempted to share with Cambridge the task of training the biochemists of the future.

In 1925, the year Hopkins received his knighthood, there appeared in '*Brighter Biochemistry*', the illustrated journal of his laboratory, an excellent picture of our 'verray parfit gentil knight' on a war horse in a full suit of armour bearing a pennon inscribed with the motto 'Gluta thy Owne': the topical allusion is obvious, but the years have brought it a deeper meaning. In his early life Hopkins felt himself able to achieve a work for science in establishing biochemistry as a separate study and in stamping it with his own mark. He fulfilled this dream of his youth by thought, work and struggle, and showed a courage and tenacity of purpose somewhat at variance with his gentle, slightly hesitating, manner.

M. STEPHENSON

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The Selective Inhibition of Cholinesterases

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In recent years it has become apparent that cholinesterase preparations from different tissues exhibit fundamental differences in specificity and other properties (Alles & Hawes, 1940; Glick, 1941; Richter & Croft, 1942). Mendel & Rudney (1943) have suggested a classification of these enzymes into two main types: (1) the 'true' cholinesterases, active only towards certain esters of choline, and (2) the 'pseudo-cholinesterases', active towards both choline and non-choline esters.

In a further paper (Mendel, Mundell & Rudney, 1943) it is stated that the 'true' cholinesterases readily hydrolyze acetyl-β-methylcholine, but are inactive towards benzoylcholine, while the reverse is true for the 'pseudo' enzyme. This difference in substrate specificity is put forward as a practical criterion for the differentiation of the two types of enzyme. From their experiments with brain and blood of different species it appears that in general brain and red blood cells contain a preponderance of the 'true' while human serum or plasma, and that of several other species, contains chiefly the 'pseudo' enzyme. Another point of differentiation was revealed in the concentrations of acetylcholine at which maximum enzymic activity was obtained, the 'true' cholinesterase showing a maximum rate of hydrolytic activity at much lower concentrations of acetylcholine than those required by the 'pseudo' enzyme (Mendel & Rudney, 1943; Bissegger & Zeller, 1943).

Studies of the action of inhibitors on these enzymes have indicated further differences. Thus, Zeller (1942) found that the cholinesterase in human or rat brain is much less sensitive to inhibition by certain pyrazolone derivatives than the cholinesterase in human serum. Hottinger & Bloch (1943) examined the action of tri-*o*-cresyl phosphate on the cholinesterase activity of rabbit and human serum, liver and brain, but found that although the human tissues were in general more sensitive to inhibition by this compound than those of the rabbit, the three tissues of either species were inhibited to approximately the same extent. Zeller & Bissegger (1943), comparing the enzymes in human serum, brain and erythrocytes, showed that whereas morphine inhibited all three esterases to approximately the same extent, percaine inhibited the enzyme in serum more powerfully than that in brain or erythrocytes, while with caffeine the reverse was true; the concentrations of inhibitor used by these latter workers were, however, high. In a study of the action of certain prostigmine analogues Hawkins & Gunter (1946) found that the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-La Roche, Nu 683) is, both *in vitro* and *in vivo*, a potent inhibitor of pseudocholinesterase, a concentration of 1×10^{-8}M producing 92% inhibition of the hydrolysis of benzoylcholine by human plasma.

Di*iso*propyl fluorophosphonate, which, like other alkyl fluorophosphonates, possesses eserine-like properties in the animal body (McCombie, Adrian, Kilby & Kilby, 1941), was shown by Mackworth (1942) to inhibit serum cholinesterase at high dilutions. Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947) have demonstrated that, while the esterase in human serum is highly sensitive to this compound, significantly higher concentrations are required to inhibit the cholinesterase in brain tissue.

It has been shown in this laboratory (Thompson, 1943, 1947; see also Gilman & Philips, 1946) that certain of the 'nitrogen mustards' (e.g. $\beta\beta'$-dichlorodiethyl-*N*-methylamine) are inhibitors of brain cholinesterase. Mackworth (1943), on the other hand, obtained only slight inhibition of the enzyme in serum. From these early results it seemed therefore as if $\beta\beta'$-dichlorodiethyl-*N*-methylamine might also be a differential inhibitor of the cholinesterases, but in the reverse sense to the fluorophosphonates, i.e. the 'true' enzyme in brain might be significantly more sensitive to inhibition by this compound than the 'pseudo' enzyme in serum. A more detailed comparison of the inhibition of certain cholinesterases by this compound has therefore been carried out with both acetylcholine and the 'specific' substrates of Mendel *et al.* (1943), and the results obtained with this inhibitor compared with those found with eserine and di*iso*propyl fluorophosphonate.

EXPERIMENTAL

Source of enzyme

Pigeon brain. Pigeons were killed by decapitation, and this was followed by immediate removal of the brain. The whole brain, wiped free from blood, was then dispersed by grinding in 10 times its weight of 0·024M-$NaHCO_3$, and filtered through muslin.

Human brain. Fresh samples of normal human cerebellum, removed during the course of operations for the excision of cerebellar tumours or chronic encapsulated abscesses, were kindly provided by Mr J. Pennybacker, F.R.C.S. After removal from the cranium the tissue was placed in ice-cold bicarbonate buffer. Large blood vessels and any adherent membranes were first dissected away, and the tissue washed free from blood clots with glass-distilled water. The weighed sample was then dispersed by grinding with bicarbonate buffer. Since appreciable quantities of blood were still present, the dispersion was strained through muslin, centrifuged, and the blood-stained supernatant liquid discarded. The precipitate was washed once more, and finally suspended in bicarbonate so that 2 ml. of the final suspension corresponded to 1 g. of original tissue.

Human plasma and serum. Freshly drawn blood was either added to potassium oxalate (1·0 mg./ml.) or allowed to clot and the clot broken up with a glass rod; separation by centrifuging followed in either case.

Human red blood cells. The cells obtained after removal of the oxalated plasma were washed five times with 0·9% NaCl, and finally lyzed by suspending in sufficient distilled water to make up to the original volume of blood.

All the enzyme preparations were stored, when necessary, at 0°, under which conditions their activity remained almost unchanged for several days. No attempt at purification was made. Plasma was used more often than serum in order to obtain more easily red cell preparations from the same blood sample. Massart & Dufait (1933*a*, *b*) have stated that oxalate is appreciably inhibitory towards horse-serum cholinesterase, due presumably to the removal of activating Ca^{++}; Gautrelet & Scheiner (1939), on the other hand, who used a biological method, have claimed that oxalate has no effect on serum cholinesterase. With human blood the final concentrations of oxalate used in this work produced less than 10% inhibition of the plasma enzyme. Further, the effects of the various inhibitors on serum or on plasma were the same, within the limits of experimental error. Consequently serum and plasma have been used interchangeably throughout the work.

Substrates

(1) *Acetylcholine chloride* (ACh) (British Drug Houses Ltd.).

(2) *Acetyl-β-methylcholine chloride* (MCh) (Savory and Moore Ltd.).

(3) *Benzoylcholine bromide* (BCh), prepared and kindly given to us by Dr A. H. Ford-Moore, Experimental Station, Porton.

Each substrate was dissolved in 0·024 M-$NaHCO_3$ immediately before use, and a sufficient volume of the solution added to the reaction mixture to bring the final concentrations to 0·006 M for ACh and BCh, and 0·03 M for MCh. Rough determinations of the Michaelis curves have shown that these concentrations lie upon the flat part of the curves obtained with the 'pseudo' enzyme, or are in excess of the peak concentrations for the 'true' enzyme (Mendel & Rudney, 1943).

Inhibitors

(1) *ββ′ - Dichlorodiethyl - N - methylamine hydrochloride* (DDM). Since this compound has been shown to undergo fairly rapid change in aqueous solution, with the formation of cyclic (ethyleneimonium) and hydroxy derivatives (Hartley, Powell & Rydon, 1942; Boyland, 1942; Cameron & Rydon, 1942; Bergmann & Fruton, 1944; Gilman & Philips, 1946), the solid was dissolved in 0·024 M-$NaHCO_3$ immediately before adding to the enzyme-bicarbonate mixture.

(2) *Diisopropyl fluorophosphonate* (DFP).

(3) *Eserine sulphate* (British Drug Houses Ltd.).

(4) *Trichlorotriethylamine hydrochloride.*

The latter inhibitors were also freshly made up in bicarbonate for each experiment.

Estimation of enzymic activity

Esterase activity was determined manometrically, using Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis of the substrate is measured by CO_2 evolution from bicarbonate buffer at pH 7·4 (gas space filled with 95% N_2 and 5% CO_2). All measurements were made in duplicate at 38°. Non-enzymic hydrolysis was controlled by including a pair of vessels containing substrate but no enzyme.

The enzyme preparation together with the inhibitor was placed in the main compartment of the Warburg vessels, and the substrate in the side bulbs, the total volume being made up to 3 ml. by the addition of 0·024 M-$NaHCO_3$ to the main compartment. After gassing the vessels were placed in the thermostat, and 15 min. allowed for temperature equilibration; the substrate was then tipped in from the side bulbs and readings taken at 10 min. intervals. The inhibitors therefore remained in contact with the enzyme, and in the absence of substrate for 5–10 min. at room temperature, followed by a further 15 min. during the attainment of temperature equilibrium.

The first 10 min. readings have been neglected and the activity recorded over the period 10–40 min. Apart from errors introduced during the removal of the manometers from the bath in order to tip in the side-bulb contents, it was found that the non-enzymic hydrolysis was usually larger during the first few minutes. Further, in the case of reversible inhibition a measurable time must elapse to allow the new enzyme-substrate-inhibitor system to approach equilibrium (Eadie, 1942).

As far as was possible the quantity of enzyme taken was adjusted to that which gave, or would have given, 180–200 μl. CO_2/30 min. with acetylcholine as substrate.

RESULTS

To test the validity of the earlier work, the effects of different concentrations of DDM were first tested on the cholinesterase activity of pigeon brain and human plasma. These tissues were chosen, largely on account of their accessibility, as sources of the true and pseudo-enzymes respectively, and experiments have been carried out using both acetylcholine and the appropriate specific substrate of Mendel. In agreement with the results of Mendel *et al.* (1943) it was found that while both preparations readily hydrolyze acetylcholine, the brain enzyme is active against MCh but not against BCh, while the reverse is true for the serum enzyme. The inhibitions of enzymic activity produced are summarized in

Table 1. *Percentage inhibition by DDM of cholinesterase activity of pigeon brain and human serum*

(See p. 171 for explanation of abbreviations here and in following tables.)

		Concentration DDM ($\times 10^{-4}$ M)							
Enzyme source	Substrate	0·17	0·44	0·87	1·75	3·5	6·1	8·7	17·5
Pigeon brain	ACh	42	69	86	93	95	—	96	—
	MCh	39	62	84	93	—	—	—	—
Human plasma	ACh	—	—	8	16	31	47	56	62
	BCh	—	11	19	28	40	—	58	—

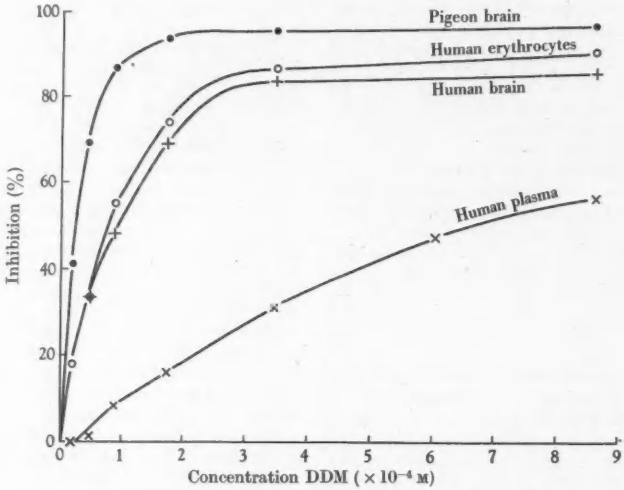

Fig. 1. Percentage inhibition of cholinesterases by DDM.

Table 1, the values for each concentration of inhibitor being the mean of several determinations, which in general agreed to within a few per cent.

The difference in the sensitivity to DDM of the cholinesterases in these two tissues is also shown in Fig. 1, from which it can be seen that, in order to produce 50 % inhibition of the enzymic hydrolysis of ACh, the concentration of inhibitor required for the plasma enzyme is about 30 times that required for pigeon brain. A similar difference exists when the specific substrates MCh and BCh are used with their respective enzymes, although appreciably more inhibition is obtained at low concentrations with the BCh-plasma enzyme system.

Since in these experiments, however, the two types of cholinesterase were derived from different animal species, the interpretation of the results is complicated by possible species differences in sensitivity to DDM. To overcome this, experiments were also carried out with preparations from human erythrocytes and human brain. The enzyme in each of these two tissues conforms to Mendel's criterion for a true cholinesterase in readily hydrolyzing ACh and MCh, while showing only a negligible activity with BCh. The results obtained with these tissues are shown in Table 2 (see also Fig. 1).

The human brain used in this work was in each experiment taken from the postero-lateral surface of the superior cerebellar lobule. Since the samples of tissue excised were not in each case identical

Table 2. *Percentage inhibition by DDM of cholinesterase in human erythrocytes and human brain*

		Concentration DDM ($\times 10^{-4}$ M)					
Enzyme source	Substrate	0·17	0·44	0·87	1·75	3·5	8·7
Human erythrocytes	ACh	18	34	55	74	86	90
	MCh	20	37	60	74	89	—
Human brain	ACh	—	34	48	69	83	85
	MCh	—	36	53	72	82	—

anatomically, considerable variations in activity were observed between them (one sample of cerebrum from the prefrontal cortex showed only negligible activity); weight for weight, the level of activity was about one eighth that of whole pigeon brain.

From Table 2 it will be seen that the activities of human erythrocytes and brain are inhibited to approximately the same extent by DDM; and although less sensitive to this substance than pigeon brain, each of these tissues is very considerably more sensitive than human plasma, 10^{-4} M-DDM causing 58 and 52 % inhibition with human erythrocyte and human brain respectively, but only 9 % with human plasma.

Differences in the response of an unpurified enzyme in different tissue preparations to the presence of a given concentration of an inhibitor might be due to variations in the amount of enzymically inert substances competing with the enzyme for the inhibitor. With this possibility in mind, and in view of the selective inhibition of the true and pseudo-cholinesterases by di*iso*propyl fluorophosphonate (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947), a series of experiments was carried out under identical conditions using DFP instead of DDM, in order directly to compare the effects of these two inhibitors on our enzyme preparations. Table 3 summarizes the results obtained with DFP on the various tissues studied.

From these results, which confirm those of Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947), it will be seen that with the same enzyme preparations functioning under the same conditions DFP inhibits selectively, but in the opposite direction to that of DDM, i.e. the plasma enzyme is very much more sensitive than that in brain to inhibition by DFP.

This contrasting behaviour of the cholinesterases from these sources is shown more clearly in Table 4, in which is given the concentrations (I_{50}) of these inhibitors which are required to produce 50 % inhibition of enzymic activity. The selective nature of the inhibitors may be conveniently presented as the ratio of these concentrations (I_{50} ratio).

In order to bring these results into line with the classical work on eserine we have also compared the effects of eserine sulphate on these enzymes under our conditions, using both acetylcholine and the specific substrates, and have confirmed the earlier findings (Mendel *et al.* 1943) that eserine shows no marked difference in its ability to inhibit the two types of cholinesterase; under our conditions 0·05 μg./3 ml. resulted in 75, 52 and 57 % inhibition of the hydrolysis of the specific substrates by the human plasma, pigeon brain and human red cell esterases respectively.

A few experiments have also been carried out with another of the 'nitrogen mustard' class of

Table 3. *Percentage inhibition of cholinesterases by DFP*

		Concentration DFP ($\times 10^{-8}$ M)									
Enzyme source	Substrate	3·6	7·1	9·1	14·4	18·2	29	36·4	59	73	144
Pigeon brain	ACh	—	11	—	19	—	30	—	52	—	85
	MCh	—	9	—	17	—	29	—	52	—	84
Human erythrocytes	ACh	8	24	—	45	—	77	—	97	—	—
	MCh	16	32	—	53	—	80	—	96	—	—
Human brain	ACh	—	—	35	—	61	—	80	—	96	—
	MCh	—	—	40	—	68	—	85	—	95	—

		Concentration DFP ($\times 10^{-8}$ M)						
		0·02	0·04	0·09	0·18	0·36	0·71	1·01
Human plasma	ACh	0	7	18	35	58	87	100
	BCh	—	—	16	34	79	86	—

Table 4. *Concentrations* (I_{50}) *of inhibitors required to produce* 50 % *inhibition of esterase activity*

Enzyme source	Substrate	I_{50} conc. of DDM ($\times 10^{-5}$ M)	I_{50} conc. of DFP ($\times 10^{-5}$ M)	I_{50} ratio (DDM/DFP)
Pigeon brain	ACh	2·4	0·057	42
	MCh	2·7	0·059	46
Human erythrocytes	ACh	7·4	0·015	493
	MCh	6·5	0·013	500
Human brain	ACh	8·7	0·013	669
	MCh	8·0	0·012	667
Human plasma	ACh	69	0·00029	$2·4 \times 10^5$
	BCh	58	0·00026	$2·2 \times 10^5$

compounds, trichlorotriethylamine hydrochloride. Although this compound is less selective than DDM in its effects on true and pseudo-cholinesterases, the enzyme in pigeon brain was again found to be more sensitive than that in human plasma.

DISCUSSION

The ability of certain compounds to inhibit the pseudo-cholinesterase at concentrations far below those required to affect the true cholinesterase has been well established. In addition to those substances already mentioned, Harris & Harris (1944) demonstrated the presence of another unidentified selective inhibitor of the pseudo-enzyme in certain curare preparations. A compound capable of inhibiting the true cholinesterase at low concentrations, while leaving the pseudo-enzyme relatively unaffected, does not, however, appear yet to have been described. Zeller & Bissegger (1943), it is true, showed that high concentrations of caffeine exerted this effect; their work, however, was carried out before the introduction of specific substrates for these enzymes, and, using a concentration of acetylcholine of 6·6 mM, they required a concentration of 6 mM-caffeine to produce 40 % inhibition of enzymic activity.

From the experiments described here, DDM, in so far as its effects on esterases are concerned, would appear to fulfil the role of a selective inhibitor of the true enzyme. It is, admittedly, a much less potent inhibitor than either DFP or eserine, but it must be remembered that its action has so far only been studied on unpurified tissue extracts. Further, few compounds of the 'nitrogen mustard' group have been even superficially investigated as cholinesterase inhibitors. It is not necessarily to be expected, therefore, that DDM is either the most potent or the most selective inhibitor among these substances. It is known that DDM also inhibits certain phosphokinases present in such tissue extracts (Dixon & Needham, 1946), so that it is not unlikely that the purified cholinesterase would show a significantly greater sensitivity to this substance. Even with our crude preparations, however, 50 % inhibition of the true cholinesterase has been obtained with concentrations varying from 2·5 to $8{\cdot}7 \times 10^{-5}$ M, whereas amounts of from roughly 10–30 times these concentrations are required to produce an equivalent inhibition of the pseudo-enzyme.

Of the large number of substances which have been described as inhibitors of cholinesterase it is now possible to distinguish three main types, examples of which are given below:

A. *Selective inhibitors for true cholinesterase:*
   ββ′-Dichlorodiethyl-*N*-methylamine.
   Caffeine.*

B. *Selective inhibitors for pseudo-cholinesterase:*
   Alkyl fluorophosphonates.
   Nu 683.
   Curare (unauthenticated samples).
   Percaine.*
   Pyrazolone derivatives.*

C. *Inhibitors of both types of cholinesterase:*
   Eserine.
   Tri-*o*-cresyl phosphate.*
   Morphine.*

* Not tested against specific substrates.

It is of interest that the values for the I_{50} concentrations of either DDM or DFP for the enzyme from a given source agree closely whether the substrate be ACh or the specific ester (Table 4), since, in the case of MCh, the concentration of substrate used differed widely from that of ACh.

The I_{50} concentrations of DDM and DFP given in Table 4 suggest strongly that, while the cholinesterase in human erythrocytes and human brain are at least very similar, they are fundamentally different from the enzyme in human plasma. Further, although the results obtained with pigeon brain differ appreciably from those obtained with the true cholinesterase in human tissues, they are in support of the general classification of the cholinesterases into true and pseudo types put forward by Mendel.

Inhibition by DFP is irreversible, and we have not found it to vary with changes in substrate concentration; DDM on the other hand appears to inhibit competitively, so that the I_{50} value will depend on substrate concentration. The I_{50} ratio for these two inhibitors will therefore also depend on substrate concentration as well as on the varying effect of enzymically inert substances present in the preparations which may be competing with the enzyme for each of the inhibitors. If, however, two more closely related inhibitors are used, interference by inert substances is less likely to affect them to different degrees. It is hoped, therefore, to extend this work to the study of two competitive inhibitors, in order to determine whether the I_{50} ratio can, under these conditions, be used as an aid in the characterization of related esterases.

SUMMARY

1. The inhibitory action of ββ′-dichlorodiethyl-*N*-methylamine on the true and pseudo-cholinesterases of brain, erythrocytes and plasma has been studied using both acetylcholine and Mendel's specific substrates, and the effects compared with those produced by di*iso*propyl fluorophosphonate and eserine.

2. ββ′-Dichlorodiethyl-*N*-methylamine has been shown to be a selective inhibitor of the true enzyme.

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The Formation of Nicotinamide from Nicotinic Acid by the Rat

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Part of the essential content of this paper has been communicated to the Biochemical Society (Ellinger, 1946*a*). The publication of details was postponed because the quantitative estimation of the nicotinamide formed, was carried out by an indirect biological method, which involved some uncontrollable factors. Confirmation of the results was desired using a more exact method which would permit the separate determination of nicotinamide and nicotinic acid in the same solution; but no suitable method has so far been found. No method based on the König (1904) principle is sensitive enough for the small amounts of nicotinamide and nicotinic acid to be measured in these experiments, and the errors involved in the estimation of nicotinamide by the differences found before and after alkaline hydrolysis are far too large. Scudi's (1946) method, based on the fluorimetric measurement of a pigment developed from nicotinamide with cyanogen bromide and alkali and extracted by *iso*butanol, permits the measurement of as little as 1 μg. of nicotinamide in the presence of nicotinic acid; the latter does not give a fluorescent product, but aneurin gives with the reagents a product of so high fluorescent efficiency that the method cannot be used for the determination of nicotinamide in the presence of aneurin. Microbiological methods using bacteria such as *Pasteurella* strains (Koser, Berkman & Dorfman, 1941) or *Leuconostoc mesenteroides* (Johnson, 1945; Krehl, de la Huerga, Elvehjem & Hant, 1946) including some strains of A.T.C.C. 9135 proved unsatisfactory, as all strains of both types of bacteria so far available responded to nicotinamide as well as nicotinic acid. It has been decided, therefore, to publish the results so far obtained.

Surviving rat liver, but not kidney or muscle, methylate nicotinamide to nicotinamide methochloride. None of the tissues mentioned converts nicotinic acid into either nicotinamide methochloride or trigonelline (Perlzweig, Bernheim & Bernheim, 1943). Ellinger & Coulson (1944) have

shown that in man two separate processes are involved in the formation of nicotinamide methochloride from nicotinic acid; these could be affected separately in certain diseases.

The present paper deals with the site and mechanism of the formation of nicotinamide from nicotinic acid by rat tissue. The only other known formation of a simple acid amide in the animal body is that of glutamine from glutamic acid (Krebs, 1935). The nicotinamide formed could only be determined by measuring the nicotinamide methochloride formed from it by liver slices, and the suitability of this method for quantitative measurement had first to be evaluated. Different rat tissues were then tested for their ability to form the amide with or without the addition of potential NH_3 donors such as glutamine or asparagine.

METHODS

Rats of both sexes, weighing 150–400 g., of black and white Lister stock or albinos of own stock, were fed after weaning on a mixed diet (Ellinger, 1946*b*); their daily urinary output of nicotinamide methochloride and their response to injected nicotinamide were studied by the method of Coulson, Ellinger & Holden (1944) in most cases. For experiment, the rats were stunned and killed by bleeding, the various organs to be examined being removed and kept in closed Petri dishes. The method of examination used was similar to that of Perlzweig *et al.* (1943). Pieces of the organs were quickly weighed, cut into slices 0·2–0·4 mm. thick, washed twice in modified Ringer's solution (Krebs & Henseleit, 1932; Krebs, 1933) and suspended in weighing bottles in 10 ml. of a similar solution containing 1 mg. of ammonium nicotinate. This solution which had previously been saturated with a mixture of O_2 (95 vols.) and CO_2 (5 vols.) was again aerated with the same gas mixture, the bottle closed securely and agitated for 3 hr. at 37°. In some experiments, slices of the organ to be examined were shaken together with liver slices, in others they were shaken separately with the ammonium nicotinate solution, the solution being decanted into another weighing bottle and liver slices added to it. After renewed aeration with the gas mixture, agitation was continued at 37° for 3 hr. In each instance the following controls were carried out. To measure the methylating efficiency of the liver used, 1 mg. nicotinamide was added instead of ammonium nicotinate to liver slices. Blanks without addition of nicotinate or nicotinamide were run, and the nicotinamide methochloride values obtained were subtracted from those of the nicotinate experiments. After shaking, the supernatant liquid was decanted into calibrated centrifuge tubes, the tissue was washed twice with 0·5 ml. of Ringer's solution, the washings being added to the supernatant liquid; 0·5 ml. of 20% (w/v) trichloroacetic acid was added, and after thorough mixing the tubes were kept in a water bath at 75–80° for 30 min. After cooling, the volume was adjusted to 12 ml. with Ringer's solution and the precipitated proteins were removed by centrifugation. In portions of the supernatant liquid nicotinamide methochloride was first estimated by the method of Perlzweig *et al.* (1943) without adsorption on Decalso and by the method of Coulson *et al.* (1944). The former frequently gave erroneously high values since the carbinols, the fluorescence of which was to be measured, were immediately oxidized into pyridones which possess much greater fluorescence efficiency than the carbinols. This method was therefore discontinued.

RESULTS

To test the suitability of the method for the quantitative estimation of nicotinamide, the following experiments were carried out. Results are described in terms of Q_{NM} defined as mg. nicotinamide methochloride formed/g. of tissue/hr.

Table 1 shows that the methylating efficiency of livers of various rats differs considerably, and that this efficiency parallels the height of nicotinamide methochloride elimination of the rat while alive. Different pieces of liver of the same rat, however, show a fairly consistent methylating efficiency, the maximal deviation being less than 10% when pieces of similar size are used. From this it is evident that the amount of nicotinamide present in a solution can be assayed when the methylating efficiency of the same liver is determined with known amounts of nicotinamide.

Table 1. *Methylating efficiency (Q_{NM}) of different pieces of the same rat liver and relation of such efficiency to the previous nicotinamide methochloride* (NM) *elimination of the rat when alive*

(Incubation 3 hr. in 5 ml. Ringer's solution with 1 mg. nicotinamide. Each mean is the result of eight experiments with liver slices weighing between 300 and 600 mg.)

No. of rat	One week's average daily NM output (μg./day)	Mean wet wt. of liver slices used (mg.)	Mean NM formed (μg.)	Mean Q_{NM}
1	57	452	5·84	4·4
		S.D. ±34·9	S.D. ±0·264	S.D. ±0·47
2	138	459	15·55	11·4
		S.D. ±36·9	S.D. ±0·902	S.D. ±0·71
3	417	438	35·6	27·0
		S.D. ±32·9	S.D. ±2·368	S.D. ±0·72
4	937	437	68·6	52·5
		S.D. ±34·8	S.D. ±4·634	S.D. ±1·31

Table 2 demonstrates that if the amount of Ringer's solution, of nicotinamide and the time of incubation are kept constant, the Q_{NM} depends on the amount of liver tissue used in the experiment, being greater with small amounts of liver and smaller with larger amounts. Between about 250 and 550 mg., the Q_{NM} is sufficiently constant to obtain comparable results.

Table 2. *Dependence of Q_{NM} of liver slices on the amount of liver used in the experiment*

(Incubation 3 hr. in 10 ml. Ringer's solution with 1 mg. nicotinamide.)

No. of rat	One week's average daily NM output (μg./day)	Wet wt. of liver used (mg.)	NM formed in 3 hr. (μg.)	Q_{NM}
5	228	53	3·6	20·4
		108	6·1	18·7
		158	8·7	18·3
		217	11·2	17·2
		263	13·3	16·8
		302	14·7	16·2
		357	17·9	16·7
		417	20·9	15·9
		480	23·5	16·3
		550	25·5	15·4
		627	29·0	15·2
		754	31·9	14·1
		877	33·6	12·8
		1012	36·9	12·2

Two more similar experiments yielded essentially identical results.

The speed of methylation was examined by incubation of pieces of the same liver (300–400 mg.) with 1 mg. nicotinamide for 0·5, 1, 2, 3 and 4 hr. and measuring the methylated product formed. The speed was fairly constant for the first 3 hr. and subsequently slowed down. An incubation of 3 hr. was therefore adopted. The influence of nicotinamide concentration on the Q_{NM} was measured by varying the amounts of nicotinamide added between 50 μg. and 5 mg. Under the conditions of the experiment, amounts increasing from 50 to 200 μg. produced linearly increasing amounts of the methylated product, but between 200 and 500 μg. a maximum of methylation was reached. The effect of pH on the methylating mechanism was examined by varying the pH (measured by glass electrode before incubation) of the Ringer's solution. The pH was varied by changing the bicarbonate concentration. Table 3 shows that the methylation of nicotinamide by liver depends on the pH and has a maximum between pH 7·35 and 7·18.

The effect of anaerobiosis on the methylating mechanism of the liver was examined either by saturating Ringer's solution with a mixture of N_2 (95 vol.) and CO_2 (5 vol.) or by adding KCN (10^{-5}, 10^{-4} or 10^{-3} M) to the Ringer's solution. Table 4 demonstrates that the methylating process is strictly aerobic and inhibited by cyanide. To examine the influence on the Q_{NM} of the thickness of the slices, these were cut 0·2–0·4 and 1–2 mm. thick from pieces of 500–550 mg. of the same liver and incubated under identical conditions. The approximate thickness was determined by cutting cubes from liver, measuring their length, and dividing by the number of slices obtained. No appreciable difference could be seen when slices of 0·2, 0·3 and 0·4 mm. were used, but slices of 1 or 2 mm. were 10–20 % less efficient.

Table 3. *Influence of* pH *on methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	Concentration of bicarbonate (M)	pH of Ringer before incubation	Wet wt. of liver used (mg.)	NM formed (μg.)	Q_{NM}	Percentage of maximum
14	0·130	8·23	371	9·4	8·4	48·1
	0·100	8·10	358	11·5	10·7	61·0
	0·070	7·87	367	14·6	13·2	75·1
	0·040	7·69	382	19·0	16·6	94·9
	0·020	7·35	353	18·3	17·3	98·8
	0·010	7·18	388	20·4	17·5	100·0
	0·005	6·76	367	15·7	14·3	81·5
	0·002	6·41	359	11·6	10·8	61·6

One other experiment yielded essentially identical results.

Table 4. *Influence of anaerobiosis and cyanide on the methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	Gas mixture	Concentration of KCN (M)	Wet wt. of liver used (mg.)	NM formed (μg.)	Q_{NM}
16	O_2/CO_2	0	396	29·4	24·7
	O_2/CO_2	0	363	27·4	25·1
	N_2/CO_2	0	372	2·2	0·2
	N_2/CO_2	0	388	0	0
	O_2/CO_2	10^{-5}	352	20·9	19·8
	O_2/CO_2	10^{-4}	393	11·9	10·1
	O_2/CO_2	10^{-3}	376	3·1	2·7

The livers used in the methionine experiment (Table 5) were chosen from various rats, whose individual spontaneous nicotinamide methochloride

elimination and whose response to injected nicotinamide had been found to differ markedly. The addition of methionine to the system nicotinamide, liver, Ringer's solution, had in many cases no effect on the methylating efficiency. However, in some instances, particularly when the initial methylating efficiency was low, methionine increased the Q_{NM} appreciably.

Table 5. *Influence of added methionine on the methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	One week's average daily NM output (mg./day)	Methionine (mg.)	Wet wt. of liver used (mg.)	NM formed (μg.)	Q_{NM}
1	57	0	443	5·6	4·2
		0	491	6·6	4·5
		1	455	10·7	7·8
		1	477	11·5	8·0
2	138	0	437	14·8	11·3
		0	485	16·8	11·5
		1	422	16·6	13·1
		1	447	17·3	12·9
3	417	0	412	34·0	26·8
		0	453	36·9	27·1
		1	423	33·3	26·2
		1	437	34·8	26·6
4	937	0	402	64·6	53·4
		0	449	71·0	52·8
		1	438	68·9	52·5
		1	453	72·8	53·6

Three more experiments yielded essentially identical results.

Liver alone synthesized nicotinamide methochloride from nicotinamide (Tables 6–8), but not from nicotinic acid (Tables 6, 7). If, however, glutamine was added to the system liver, ammonium nicotinate, Ringer's solution, some formation of nicotinamide methochloride could be observed in about half of the experiments, the addition of asparagine having no effect (Table 6). The glutamine used was tested before use with Nessler's reagent (Archibald, 1944).

Table 6. *Influence of added glutamine* (Gl) *or asparagine* (As) *on the formation of nicotinamide methochloride* (NM) *from* 1 *mg. of ammonium nicotinate* (Am.nic.) *by liver slices*

(Incubation 3 hr.)

No. of rat	Added Gl or As (mg.)	Substrate	Wet wt. of liver slice (mg.)	NM formed (μg.)	Q_{NM}
22	0	Nicotinamide	433	24·5	18·8
	0	Nicotinamide	448	25·9	19·3
	0	Am.nic.	471	0·3	0·2
	0	Am.nic.	406	0	0
	10 Gl	Am.nic.	423	0	0
	10 Gl	Am.nic.	434	3·0	2·3
	10 Gl	Am.nic.	444	11·1	8·3
	10 Gl	Am.nic.	412	11·9	9·6
	10 As	Am.nic.	433	0·1	0·1
	10 As	Am.nic.	428	0·4	0·3

Four more experiments yielded essentially identical results.

The following organs were tested for amidation of nicotinic acid: kidney cortex, brain cortex, spleen,

Table 7. *Synthesis of nicotinamide methochloride* (NM) *from ammonium nicotinate* (Am.nic.) *and nicotinamide* (NA) *by slices of various organs*

(Incubation 3 hr.)

Tissue	No. of experiments	Limits of wt. of liver (mg.)	Substrate	Limits of wt. of tissue other than liver (mg.)	NM formed/g. liver/hr. (μg.) (average in brackets)
Liver	43	230–610	NA, 1 mg.	—	3·6–73·5 (27·3)
Liver	22	290–580	Am.nic., 1 mg.	—	0–2·9 (0·5)
Kidney	7	—	NA, 1 mg.	230–320	0–2·1 (0·5)
Kidney	7	—	Am.nic., 1 mg.	220–340	0–1·2 (0·3)
Brain	8	—	NA, 1 mg.	180–280	0–1·9 (0·3)
Brain	8	—	Am.nic., 1 mg.	170–300	0–0·8 (0·2)
Liver	7	354–560	Kidney + NA, 1 mg.	140–590	4·3–58·2 (26·4)
Liver	30	340–590	Kidney + Am.nic., 1 mg.	255–585	4·1–62·3 (22·1)
Liver	8	360–580	Kidney + Am.nic., 1 mg. (anaerobically)	330–470	0–1·3 (0·3)
Liver	7	360–440	Brain + NA, 1 mg.	130–190	7·2–59·2 (26·1)
Liver	20	380–520	Brain + Am.nic., 1 mg.	120–210	7·6–51·3 (21·7)
Liver	4	365–490	Brain + Am.nic., 1 mg. (anaerobically)	140–190	0–1·1 (0·3)
Liver	4	330–430	Spleen + Am.nic., 1 mg.	165–225	0
Liver	4	320–480	Skelet. muscle + Am.nic., 1 mg.	530–620	0
Liver	4	330–450	Heart muscle + Am.nic., 1 mg.	260–300	0
Liver	4	350–450	Pancreas + Am.nic., 1 mg.	50–70	0–0·9 (0·3)
Liver	4	380–510	Intestin. mucous membrane + Am.nic., 1 mg.	110–130	0–0·8 (0·2)

skeletal muscle, heart muscle, pancreas and intestinal mucosa. In each case slices were incubated with 1 mg. of ammonium nicotinate and liver slices, and parallel experiments were made with nicotinamide instead of nicotinate. In addition, the formation of nicotinamide methochloride from ammonium nicotinate or nicotinamide by kidney or brain slices without liver was tested. Table 7 shows that, of all tissues tested, only kidney and brain cortex were able to form nicotinamide from ammonium nicotinate. In some of the experiments, the pyridine carbinols were immediately oxidized to pyridones when estimated by the method of Coulson *et al.* (1944) when kidney was agitated together with liver. No measurement was possible owing to the different fluorescence efficiencies of the

Table 8. *The influence of disintegration of liver tissue on the methylation of nicotinamide and of kidney and brain on the amidation of ammonium nicotinate* (Am.nic.)

(Incubation 3 hr.)

No. of rat	Tissue	Wet wt. of liver used (mg.)	Substrate	NM formed (μg.)	NM formed/g. liver/hr. (μg.)
27	Liver slices	354	1 mg. NA	24·8	23·3
	Liver slices	377	1 mg. NA	26·8	23·7
	Liver broken up cells	348	1 mg. NA	0·2	0·2
	Liver broken up cells	386	1 mg. NA	0	0
	Liver slices	349	1 mg. Am.nic.—302 mg. kidney slices	18·6	17·8
	Liver slices	362	1 mg. Am.nic.—312 mg. kidney slices	19·8	18·2
	Liver slices	380	1 mg. Am.nic.—331 mg. kidney broken up cells	0·4	0·3
	Liver slices	337	1 mg. Am.nic.—347 mg. kidney broken up cells	0·1	0·1
	Liver slices	367	1 mg. Am.nic.—173 mg. brain slices	17·8	16·2
	Liver slices	358	1 mg. Am.nic.—194 mg. brain slices	18·8	17·4
	Liver slices	371	1 mg. Am.nic.—167 mg. brain broken up cells	0·1	0·1
	Liver slices	349	1 mg. Am.nic.—203 mg. brain broken up cells	0	0

Table 9. *Influence of* pH *on amide formation from ammonium nicotinate by kidney and brain slices*

(Incubation 3 hr., 1 mg. ammonium nicotinate.)

No. of rat	Concentration of bicarbonate (M)	pH of solution before incubation	Wet wt. of liver (mg.)	Wet wt. of kidney (K.) or brain (B.) (mg.)		NM formed (μg.)	Q_{NM}	Percentage of maximum
28	0·130	8·23	356	259	K.	8·4	7·9	37·2
	0·100	8·10	349	254		11·3	10·8	51·0
	0·070	7·87	377	263		16·1	14·3	67·4
	0·040	7·69	383	258		20·8	18·3	86·0
	0·020	7·35	354	255		22·0	20·7	97·3
	0·010	7·18	392	260		25·1	21·3	100·0
	0·005	6·76	351	257		22·7	19·8	93·2
	0·002	6·41	366	256		15·1	13·7	64·5
	0·130	8·23	348	202	B.	7·1	6·8	30·1
	0·100	8·10	352	207		10·0	9·5	49·4
	0·070	7·87	361	203		13·8	12·7	66·0
	0·040	7·69	354	208		17·0	16·1	83·7
	0·020	7·35	357	204		20·2	18·9	98·0
	0·010	7·18	349	203		20·2	19·3	100·0
	0·005	6·76	360	207		19·5	17·7	92·8
	0·002	6·41	352	202		13·6	12·9	67·0

two compounds and these experiments had to be discarded. The mechanism of amidation by kidney and brain was studied under anaerobic conditions by using a mixture of N_2 (95 vol.) and CO_2 (5 vol.) for saturating and aerating Ringer's solution in which the kidney or brain were incubated. Before adding the supernatant liquid to the liver slices, it was thoroughly aerated with the O_2-CO_2 mixture for 15 min. The methylating process was strictly aerobic.

Broken up cells (*brei*) of liver, kidney and brain were also tested for their amide-forming and methylating activities. They were prepared by grinding the finely cut tissue in an agate mortar. Table 8 shows that the methylating effect of the liver as well as the amidation by kidney or brain is bound up with the intact structure of the cell.

To investigate the dependence on pH of the amidation by kidney or brain, slices of these organs were incubated in Ringer's solutions of various pH (glass electrode) prepared by using varying amounts of bicarbonate. In order, however, to avoid an effect of altered pH on the methylation by liver, the supernatant liquid after incubation was adjusted to pH 7·2 by addition of bicarbonate or hydrochloric acid solutions (external indicators) before it was incubated with the liver slices. The amounts of kidney or brain used in this experiment differed from each other by less than 10%. Table 9 shows that the amide formation by kidney and brain depends on the pH. The maximum in both cases lies between pH 7·35 and 6·76.

DISCUSSION

Examination of the conditions under which liver slices methylated nicotinamide showed the reaction to be suitable as a qualitative test for nicotinamide and, under strictly controlled experimental conditions, also to provide a quantitative measure of small amounts of nicotinamide in the presence of nicotinic acid. The findings of Perlzweig *et al.* (1943) were confirmed that liver, but neither kidney nor muscle, methylated nicotinamide, that methionine increased the methylation in some cases, that methylation proceeded at a fairly constant speed for about 3 hr., that a maximum effect was reached by amounts of 250 μg. nicotinamide/350–400 mg. of liver, that the process is aerobic and requires intact cells; but, contrary to the findings of Perlzweig *et al.* (1943), it was found that different pieces of liver of the same rat were of the same methylating efficiency. Perhaps their 'somewhat erratic results' are due to their method of assay without adsorption and elution. Some information could be obtained concerning properties of the enzymic system responsible for nicotinamide methylation such as dependence on pH, inhibition by anaerobic conditions and HCN, and dependence on intact cell structure. The specificity of the enzyme for nicotinamide is demonstrated by the observation of Perlzweig *et al.* (1943) that after incubation of nicotinic acid with liver slices no trigonelline formation was observed with the method of Sarett (1943), and by Ellinger, Fraenkel & Abdel Kader's findings (1947) that none of a large number of nicotinamide derivatives was methylated by the enzyme.

Of all organs tested for their ability to convert nicotinic acid into nicotinamide, only kidney and brain were found active. These are the same organs which were shown by Krebs (1935) to form glutamine from ammonia and glutamic acid; retina was not examined in the present experiments. A few properties of the nicotinic acid amidase were investigated, such as pH activity curve and dependence on the presence of oxygen and intact cell structure. The specificity could not be tested; this can only be done when an exact method for separate estimation of nicotinamide and nicotinic acid becomes available.

Certain difficulty attended the construction of a pH activity curve for the nicotinic acid amidase, since the nicotinamide formed could be measured only indirectly by means of two separate biological processes with all their attendant inaccuracies. Unnecessary corrections were eliminated as far as possible by using constant amounts of kidney and brain.

No explanation can be given at present for the peculiar effect of glutamine on the conversion of nicotinic acid into nicotinamide methochloride by liver tissue, or for the finding that this effect occurs only on some occasions.

SUMMARY

1. The methylation of nicotinamide by liver tissue *in vitro* has been studied and found to be suitable as a qualitative, and with certain restrictions as a quantitative assay method for nicotinamide in the presence of nicotinic acid. The conditions of this process have been examined and some properties described of the enzyme system involved.

2. Kidney and brain have been found to convert nicotinic acid into nicotinamide *in vitro*. Some of the properties of the enzyme system involved in this amide formation have been studied.

3. Liver did not convert nicotinic acid into nicotinamide methochloride *in vitro*, but when glutamine was added formation of nicotinamide methochloride from nicotinic acid occurred in some instances.

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Electrophoretic Studies on Human Serum

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The purpose of the present work was to collect information of the extent to which the interpretation of electrophoretic patterns of serum proteins in phosphate buffer can account for 'boundary anomalies', an expression used by Tiselius (1930) and others to refer to small changes in field intensity and mobility across a boundary.

Although the nature of the changes in conductivity and ionic mobility across a protein boundary has been recognized for some time (Tiselius, 1930; Longsworth & MacInnes, 1940*a*; Abramson, Moyer & Gorin, 1942), it is only recently that these changes have been given a detailed theoretical consideration (Svensson, 1943; Dole, 1945; Svensson, 1946). A theoretical treatment is here given which incorporates Svensson's conclusions on boundary anomalies and has been used to study in particular (*a*) the relative migration velocities of the α_2 and A boundaries in human serum, and (*b*) the conditions during electrophoresis of solutions containing protein components of nearly the same mobilities.

THEORETICAL

Notation

The symbols used are defined as follows:

V_i = migration of protein i (numbered from the fastest component) at infinite dilution in the buffer of concentration equal to that immediately below the ϵ boundary;

V_{ai} = actual migration velocity of protein i below its boundary in descending limb;

r = ratio of velocities of the protein ions at infinite dilution in the bulk buffer to that in buffer below ϵ boundary;

C_i = apparent concentration of protein i on descending side $= \dfrac{\text{area under peak } i}{\text{total area desc. } -\epsilon} \times$ concentration of protein in starting solution;

C_1' = apparent concentration of protein i on ascending side $= \dfrac{\text{area under peak } i}{\text{total area asc. } -\delta} \times \rho \times$ concentration of protein in starting solution;

C_i and C_1' are expressed in g./100 ml.;

$\rho = \dfrac{\text{mean of asc. and desc. total areas } -\delta}{\text{mean of asc. and desc. total areas } -\epsilon}$;

a_i, a_i' = boundary displacements on the descending and ascending sides during an arbitrary time;

K_i = coefficients as defined in the text;

κ = conductivity;

I = current per unit area of cross-section.

Notation for Svensson's (1946) *equation* (23)

c_{i1} = ionic concentrations in electrochemical equiv./ml. above colloid boundary, with the signs of the charges;

u_{i1} = ionic mobilities in cm.^{2} V.$^{-1}$ sec.$^{-1}$ above colloid boundary, with the signs of the charges;

u_2/u_1 = mobility ratio across the colloid boundary;

C = concentration of a leading ion in equiv./ml. with the sign of the charge;

$\mathbf{U}$ = mobility of colloid ion above the boundary, defined by equation (22) of Svensson (1946); $\mathbf{U} = \overline{U}$ of Svensson;

κ_2/κ_1 = ratio of conductivities of solutions above and below the colloid boundary.

The displacement of the centroidal ordinate of the gradient curve of a boundary gives the migration velocity of the protein ion in the environment below its boundary (Longsworth, 1943). If the relative change in migration velocity of a protein ion, brought about by a change in the

concentration of protein from zero to C_i is put proportional to the protein concentration, i.e. $(V_{ai} - V_i)/V_i = K_i C_i$, then the velocity of, for example, the second slowest descending boundary is given by $V_{i-1}(1 + K_i C_i + K_{i-1} C_{i-1})$, that of the fastest ascending boundary by $rV_1(1 + K_1 C'_1)$. Generally:

$$V_1(1 + K_i C_i + K_{i-1} C_{i-1} + \ldots + K_3 C_3 + K_2 C_2 + K_1 C_1):$$
$$V_2(1 + K_i C_i + K_{i-1} C_{i-1} + \ldots + K_3 C_3 + K_2 C_2): \ldots:$$
$$V_i(1 + K_i C_i) : rV_1(1 + K_1 C_1') : rV_2(1 + K_1 C_1' + K_2 C_2'): \ldots:$$
$$rV_i(1 + K_1 C_1' + K_2 C_2' + \ldots + K_i C_i')$$
$$= a_1 : a_2 : \ldots : a_i : a_1' : a_2' : \ldots : a_i'. \qquad (1)$$

The coefficients K_i are intended to account for both the increased field strength and the changed mobility below the protein boundary.

The conditions necessary for these simple proportionality relations to hold can be derived from the equation for the conductivity change across a boundary given by equation (23) of Svensson (1946):

$$\frac{\kappa_2}{\kappa_1} = \frac{u_2}{u_1}\left[1 - \frac{C}{\Sigma \frac{c_{i1} u_{i1}}{u_{i1} - \mathbf{U}}}\right].$$

This implies

$$-\frac{\Delta\kappa}{\kappa_1} + \frac{\Delta u}{u_1} = \frac{u_2}{u_1} \frac{C}{\Sigma \frac{c_{i1} u_{i1}}{u_{i1} - \mathbf{U}}}. \qquad (2)$$

However,

$$-\frac{\Delta\kappa}{\kappa_1} + \frac{\Delta u}{u_1} = \frac{(V_{ai} - V_i)\kappa_2}{V_i \kappa_1}$$

for a protein i, where

$$V_{ai} = \frac{u_{i2} I}{\kappa_2} \quad \text{and} \quad V_i = \frac{u_{i1} I}{\kappa_1}, \qquad (3)$$

u_{i2}/u_{i1} of the protein ions being taken equal to u_2/u_1. After elimination of κ_2/κ_1,

$$\frac{V_{ai} - V_i}{V_i} = \frac{C}{\Sigma \frac{c_{i1} u_{i1}}{u_{i1} - \mathbf{U}} - C}. \qquad (4)$$

The conditions for equation (1) to hold are then (*a*) that C be small compared with $\Sigma \frac{c_{i1} u_{i1}}{u_{i1} - \mathbf{U}}$, $K_i = \frac{1}{V_i} \frac{V_{ai} - V_i}{C_i}$ being then an approximation to $\left(\frac{1}{V_i} \frac{dV_{ai}}{dC_i}\right)_{c_i \to 0}$ at constant buffer salt concentration in the supernatant fluid, and (*b*) that u_{i2}/u_{i1} for the protein ions be approximately equal to u_2/u_1 for the buffer ions. It is considered that these conditions are satisfied in the experiments described in the present paper on account of the small specific charge of the protein ions, and since the changes in pH across the protein boundaries could be assumed to be negligible. If other protein components are present on both sides of the protein boundary, the V_i refer to the velocity in buffer in the absence of the other protein ions as stated in the definition. The use in equation (1) of the apparent concentrations, where strictly the true concentrations are concerned, was taken as an approximation for cases in which the concentrations of the protein components on both sides of protein boundaries could be assumed to differ little. When two components have very similar mobilities, the true concentrations must be used in the calculation, as is done in a later section of this paper. Equation (1) has been derived for a special purpose for which an extended treatment was not considered necessary, the advantages conferred by simplicity outweighing the limitations due to the imposed conditions.

EXPERIMENTAL

The electrophoresis experiments were carried out in the Tiselius apparatus fitted with the Philpot-Svensson diagonal edge optical system. In place of the diagonal edge a wire of 0·19 mm. diameter was used. The technique of electrophoresis has been described by several authors (see Svensson, 1946; Longsworth, 1946; Wiedemann, 1946). As can be seen from the base lines in the accompanying photographs, the optical system used has not the perfection of spherical correction attainable by more recent developments. It was tested for inadequacies not obvious in the appearances of the base lines by measuring the areas given by salt boundaries which had been moved to four different positions on each limb, and it was found that the range of variation in the values for the areas ($\pm 1{\cdot}5\%$) did not appreciably exceed the experimental error. The optical system was also tested by comparing the patterns given by a lens (one lens of focal length 200 cm. and another of focal length 25 cm.) placed at different positions in a vertical plane at the place of the **U**-tube. A mm. scale, placed between the limbs of the **U**-tube, gave a practically undistorted image (without the cylindrical lens; within $\pm 0{\cdot}05$ mm. in all and within $\pm 0{\cdot}03_5$ mm. in 31 out of 34 pairs of measurements).

Serum was diluted with phosphate buffer of pH 8·0 and ionic strength $\mu = 0{\cdot}1$, and dialyzed against 2 l. of buffer in the refrigerator at 0–4° for several days with frequent mixing. The **U**-tube was of the medium-size type requiring about 10·5 ml. to fill the bottom compartments and the bridge. Currents between 3 and 20 mA. were used (cross-section of the **U**-tube = 75 sq.mm.). The bath temperature was 0·1°. The base lines were photographed before each experiment was started, after the bottom compartment had been set in alignment. The areas were measured with a planimeter on enlarged tracings. The method of dividing the areas was that of Svedberg & Pedersen (1940) as used by Longsworth (1946). For grossly asymmetric curves, like those of the β and γ peaks, curves of different steepness were used for the extrapolation of each side. The small component behind the β-globulin was always included in the γ-globulin. The displacements of the albumin and the α-globulin boundaries only were measured, since the position of the β-globulin boundary on the descending side is disturbed by the presence of the β-anomaly, and the relative migration velocity of the γ peak is too small for the accuracy of measurement required. The area of the β-globulins, however, can be obtained correctly from records taken 10–120 min. after the current has been stopped, when the anomalous part of the curve has shrunk to within the measurable range. Any material flowing off or collecting in the β boundary should, according to Moore & Lynn (quoted by Abramson *et al.* 1942, p. 186), not alter the value for the area underneath the β-globulin peak. The difference in the displacements of the A and α boundaries was determined from measurements of the centroidal ordinates of the gradient curves (Longsworth, 1943; Dole, 1945) to an accuracy of 0·3 mm. or better. Ordinates at intervals of 0·2 mm. were placed parallel to the streaks on the pattern (originating from dust particles in focus on the plate), and

the position of the centroidal ordinate was obtained by $\mathbf{x}=\Sigma x_i y_i/\Sigma y_i$, where x_i is the distance in the direction of migration and y_i that along the ordinate. While it was possible to locate the A boundaries in the descending limb to within $\pm 0{\cdot}1$ mm. or better, the location of the α boundary was often made more uncertain since an appreciable part of the curve was obtained by extrapolation. The centroidal ordinates for the ascending albumin boundaries at concentrations higher than 1 g./100 ml. were obtained by extrapolating the top part of the gradient curve which fell outside the range covered by the photographic record. The area of this part of the peak was calculated by difference of the total area of the ascending A peak and that of the available part, the total area of the ascending A peak from that of the descending A peak by multiplication by ρ, and ρ was obtained as defined above assuming the total areas under all peaks (including δ and ϵ) to be equal on both sides. All these values are crude approximations, but errors in neither of them affect the position of the centroidal ordinate significantly. If the missing part of the curve is taken to be symmetrical with respect to an ordinate half way between the highest (equal) ordinates measured, then the same value for the centroidal ordinate is obtained, independent of the shape of this part of the curve, which may be between that of an equilateral triangle and a rectangle. The most probable shape of the missing part of an asymmetrical curve will be between that of a rectangle and that of a triangle, the sides of which are in the same ratio as the areas under the curve outside the highest ordinates measured, but nearer that of the latter. The differences in the positions of the centroidal ordinates for the extreme cases were in the present experiments between 0·016 and 0·033 mm. The position of the centroidal ordinate used in the calculation for Exps. 3 and 7–10 of Table 1 was that calculated for the missing part of the curve as an asymmetrical triangle.

The displacements were measured from the position of the original boundary, and no correction was applied for volume changes due to the electrode reactions (Longsworth, 1943; Dole, 1945).

RESULTS

The relative migration velocities of the α_2 and A boundaries

For a system of four components such as that of the four groups of proteins in human serum (phosphate buffer) the appropriate equations are:

$$V_1(1+K_4C_4+K_3C_3+K_2C_2+K_1C_1):$$
$$V_2(1+K_4C_4+K_3C_3+K_2C_2):rV_1(1+K_1C_1'):$$
$$rV_2(1+K_1C_1'+K_2C_2')=a_1:a_2:a_1':a_2'. \quad (5)$$

They can be solved for r and the K_i if all concentrations and displacements are known and if a relationship is assumed between the coefficients K_i. For the present purpose the values of K_1 and r were calculated from (5) by assuming K_4, K_3 and K_2 to be equal to K_1. The mean values from both patterns of the apparent relative concentrations (assuming, as is usual, equal refractive indices for all components), multiplied by the protein concentration of the starting solution, were taken as the values for the apparent concentrations on the descending side. The concentrations of albumin and α_2-globulin on the ascending side were calculated from the mean values by multiplication by ρ as the best approximation to the values of the apparent concentrations. Where the area of the albumin peak on the ascending side could not be measured, the areas on the descending pattern only were used for calculation. The area of ϵ, which could in no serum pattern be defined with any certainty, was taken as one fifth of δ. The ratio ϵ/δ for the equilibrated solution of albumin in Fig. 1A and B was found to be $0{\cdot}2_7$. On account of the nature of equation (5) there was no necessity for any great accuracy in the location of the position of the original boundary, provided all measurements on both sides were taken from points equidistant from the centre line between the partition plates. In order to make use of patterns where no record of the position of the original boundary had been taken, values for K_1 and r were bracketed within the limits obtained by the use of the distances of the boundaries from the δ and the descending γ boundaries respectively.

Table 1 shows the results obtained from ten electrophoresis experiments on normal and pathological sera. The values for r incorporate the ratio of the cross-sections of both sides of the **U**-tube, for which no correction has been applied. They are, furthermore, much dependent on the assumed relationship between the K_i and they do not, therefore, represent an experimental approach to obtaining the ratio of velocities as stated in their definition, but merely an aid for obtaining values for K_i, in the calculation of which they are made to cancel out.

The coefficient K_1 was about 0·1 in all experiments, signifying an increase of about 10 % in the migration velocity for an increase of 1 g./100 ml. in the protein concentration. Such a high value was rather unexpected, and therefore the effects on K_1 of the uncertainty involved in the values employed for ρ and δ were tested by repeating the calculation, putting in their place $\frac{1}{2}(\rho+1)$ or $\frac{1}{2}\delta$. (The altered value of ρ was used with the same relative concentrations of the components, while that of δ involved a change in the relative concentrations, mainly in that of γ-globulin.) For example, using the value $\frac{1}{2}(\rho+1)$, K_1 in Exp. 3 was then found to be lowered by 0·001. Using $\frac{1}{2}\delta$ in Exp. 2 the same value for K_1 was found. By taking $K_4=K_3=K_2=0$ the value for K_1 in Exp. 10 was increased by 0·0009. By taking $K_4=K_3=K_2=2K_1$ the value for K_1 was 0·0026 lower; that for K_2, etc., worked out to 0·19, which is improbably high. Thus a drastic change in the assumptions about the coefficients or errors in the estimates of ρ or δ did not alter K_1 to any considerable extent.

Another estimate of K_1, not involving proteins other than albumin, was therefore obtained by comparing the distances migrated by the ascending albumin boundaries at different concentrations of serum. Serum was diluted to a total protein content of 0·61, 1·17 and 2·38 g./100 ml., and all three samples dialyzed together against 2 l. of buffer. The three electrophoresis experiments on them (Exps. 1, 2 and 3 of Table 1) were carried out on one day against the same supernatant fluid and without change of the liquids in the electrode vessels.

Table 1. *Coefficient K_1 derived from the relative migration velocities of the α_2 and A boundaries*

Exp. no.	Diagnosis	Conc. of protein (g./100 ml.)	Albumin ($+\alpha_1$) (% of total)	K_1	r
1	Normal	0·6	65	0·21	1·12
2		1·2		0·12	1·17
3		2·4		0·12	1·32
				(δ as origin) ($\gamma_{desc.}$ as origin)	(δ as origin) ($\gamma_{desc.}$ as origin)
4	Normal	1·4	62	0·08—0·12	1·13—1·18
5	Normal	1·5	63	0·11—0·15	1·20—1·27
6	Plasma-cell myeloma	1·9	50	0·13—0·17	1·29—1·36
7	Breast carcinoma	2·4	70	0·07—0·11	1·23—1·29
8	Breast carcinoma	2·5	58	0·09—0·12	1·28—1·35
9		2·5	60	0·10—0·14	1·33—1·42
10	Cirrhosis of liver	2·5	57	0·09—0·13	1·30—1·36

Table 2. *Migration velocities of albumin boundaries and apparent composition of serum (in g./100 ml.) at different concentrations*

(Phosphate buffer pH 8·0, $\mu = 0{\cdot}1$, 120 min. at 12·0 mA.)

Concentration of total protein (g./100 ml.)		0·61			1·17			2·38			1·4*		
Distances migrated (mm.)	Ascending side	28·$_2$			29·$_3$			30·$_0$			—		
	Descending side	26·$_4$			26·$_2$			25·$_6$			—		
		Asc.	Desc.	Mean	Asc.	Desc.	Mean	Asc.	Desc.	Mean	Asc.	Desc.	Mean
Albumin ($+\alpha_1$)		4·47	4·35	4·41	4·65	4·37	4·51	—	4·56	—	4·52	4·47	4·50
α_2		0·50	0·57	0·53	0·33	0·50	0·41	—	0·44	—	0·60	0·43	0·54
β		0·92	0·88	0·90	0·84	0·89	0·87	—	0·90	—	0·83	0·95	0·89
γ		1·01	1·10	1·06	1·08	1·14	1·11	—	1·00	—	0·95	0·99	0·97
Albumin ($+\alpha_1$) + α_2		4·97	4·92	4·94	4·98	4·87	4·92	—	5·00	—	5·12	4·96	5·04
$\frac{\delta \text{ (or } \epsilon)}{\text{Total area asc.}}$		—	—	—	0·08	—	—	0·16	—	—	0·11	0·055	—

* Veronal buffer pH 8·6 as used by Longsworth, Curtis & Pembroke (1945).

Table 2 shows the distances migrated in equal times at equal currents. They were measured from the position of the original boundary.

The increase on the ascending side in the distance migrated by the albumin boundary is 6 % for an increase in the albumin concentration (corrected for dilution in the ascending limb) of about 1 g./100 ml. The coefficient K_1 therefore works out to be 0·06. The results of the area measurements are included to show the effect of the concentration on the apparent relative concentrations of the components. The sum of $A(+\alpha_1)+\alpha_2$ should be used for comparison on account of the uncertainty involved when extrapolating the curve between $A(+\alpha_1)$ and α_2. The change in the apparent concentration of $A(+\alpha_1)+\alpha_2$ for the whole practicable range is about 2 % in the descending patterns, but this is here within the experimental error. Perlmann & Kaufman (1945) found a change in the proportion of the albumin to total protein, as calculated from the areas given by human plasma in veronal buffer, from 58·4 to 54·6 % as the total protein concentration was changed from 2·5 to 0·94 g./100 ml. (4 to 1·5 mg. N/ml.). The change in this case is about 6·5 %.

Simultaneous measurements of velocities at different concentrations. Small differences in migration velocities could be measured more satisfactorily in simultaneous experiments than otherwise; one such experiment is reported here in detail, since the technique leads to a new method of comparing mobilities. The method was based (*a*) on the theoretical consideration that the δ boundary should be completely absent when the starting solution is a solution of a single component obtained from between the ascending boundary and the δ boundary and when the buffer used as supernatant is the same as that into which the protein component has migrated previously (Lagercrantz, 1945), and (*b*) on the assumption that for low concentrations of the protein the changes in buffer-ion concentrations across the ascending boundary are proportional to the protein concentration.

Experimental evidence for (*a*) was obtained during a preparation of serum albumin. An albumin solution (18 ml.) free from α_1 and α_2 was prepared by several electrophoretic runs on undiluted serum dialyzed against veronal buffer (pH 8·6). The pooled samples were then electrophoresed into veronal

buffer and they proved to be free from α_2 or α_1. This solution was then dialyzed with frequent stirring against four changes of 2 l. each of phosphate buffer of pH 8·0 during 12 days. Electrophoresis into phosphate buffer gave the patterns as shown in Fig. 1 A and B. Apart from the albumin boundary and a

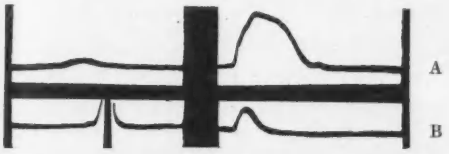

Fig. 1. Electrophoretic patterns of serum albumin free from α_1- and α_2-globulin. A, ascending albumin and δ, after 57 min. at 20 mA., tilt 60° to the horizontal; B, descending albumin and ϵ, after 92 min. at 20 mA., tilt 60°; phosphate buffer pH 8·0, $\mu = 0{\cdot}1$. The direction of migration is to the left for ascending and to the right for descending patterns.

minute component ahead of it, only the δ and ϵ peaks are seen. The area of the δ peak amounted to 16 % of the total area (protein concentration 1·8 g./100 ml.). This electrophoresis was continued for 6·5 hr. at 20 mA., while the ascending boundary was kept in position by steady compensation, and there was no disturbance by convection on the ascending side, whereas on the descending side convection began when the δ boundary had reached the bridge. The albumin solution from the ascending side, which was expected to show no δ effect, was separated. The remainder of the solution was mixed with the solution, dialyzed against phosphate in the ratio 3 : 2 and electrophoresed (with an interruption of 11 hr.) for 12 hr. at 20 mA. The δ effect was now smaller than in the previous run, since a greater part of the starting solution had had the buffer salt concentration reduced during the previous experiment. This run also lasted much beyond the time at which the δ boundary passed the bridge, and thus the concentration in buffer salts on the descending side must have been further reduced, approaching that on the ascending side. The final run was made with a solution consisting of 2 parts of albumin solution from the ascending side, 1 part of the remainder from the bridge and descending side, and a small amount of buffer to make up the volume required. A small δ peak was observed, the area of which indicated a change in refractive index of 1·7 % of that at the descending A boundary.

If the assumption that the change in migration velocity at a protein boundary is proportional to the change in protein concentration holds in the range of concentrations used, then no gravitational instability should arise, and no additional δ boundary should appear in the experiment carried out as follows: The layer formed by the electrophoresis of serum between the first two boundaries on the ascending side, A and α_2, was recovered from the **U**-tube, and diluted with buffer to reduce the concentration as much as possible consistent with retaining a distinct boundary peak. This dilute protein solution was then used as supernatant in place of the buffer in the top compartment on the ascending side in the next electrophoresis of the same serum. In both runs portions of the same dialyzed serum and the same buffer against which it has been dialyzed were used. The interference of α_1 can be disregarded, since its concentration in the solution of the diluted albumin is negligible and the difference in mobilities between A and α_1 in phosphate buffer is small.

The difference between the migration velocity of a boundary of a concentrated and that of a diluted albumin solution can be measured directly by the change in the distance between these two boundaries. Table 3 gives the experimental details, and Fig. 2 shows the patterns of an experiment carried out with normal serum. Assuming that the δ boundary has moved 2·5 mm. backward with reference to the position of the original boundary as was observed to be the case in Exp. 3 (Table 1), the distance migrated by the $A_{\text{conc.}}$ boundary in 145 min. was 33 mm. The change in the distance $A_{\text{conc.}} - A_{\text{dil.}}$ was 2·1 mm. and the distance migrated by the $A_{\text{dil.}}$ boundary 30·9 mm. The velocity of the large albumin boundary does not represent the migration velocity of the albumin ions since these ions are present on both sides of the boundary (Svensson, 1946). The difference between the velocity of the boundary, and the true velocity of the albumin ions behind the boundary, can be estimated in the present case in a simple manner on account of the assumption that a change in the concentrations of any ion species cannot occur either behind the $A_{\text{dil.}}$ or between the $A_{\text{conc.}}$ and the α_2 boundary because δ boundary effects, apart from those normally present, are bound to be absent in these regions. Owing to the difference in velocity of the $A_{\text{conc.}}$ and $A_{\text{dil.}}$ boundaries the volume/unit cross section occupied by the dilute albumin solution has been reduced by $rV_1(K_1C'_{\text{conc.}} - K_1C'_{\text{dil.}})$. The amount of albumin in the volume $rV_1(K_1C'_{\text{conc.}} - K_1C'_{\text{dil.}})$ is $rV_1K_1(C'_{\text{conc.}} - C'_{\text{dil.}})\,C'_{\text{dil.}}$. This amount appears in the layer of concentrated albumin, the volume of which (per unit cross section) is increased by

$$\frac{rV_1K_1(C'_{\text{conc.}} - C'_{\text{dil.}})\,C'_{\text{dil.}}}{C'_{\text{conc.}} - C'_{\text{dil.}}}.$$

The length (= volume/unit cross section) of the layer of concentrated albumin is therefore greater by this amount than it would have been if the concentrated

Table 3. *Relative migration velocities of concentrated and diluted serum albumin*

Time (min.)	Current (mA.)	Distances in mm. on plate: $A_{conc.} - \delta$	$A_{conc.} - \gamma_{asc.}$	$A_{desc.} - \gamma_{desc.}$	$A_{conc.} - A_{dil.}$	Fig.
(I) 145	10	35·5	28·4	24·4	—	—
(II) 0	—	—	—	—	50·9	A
72	10	—	—	—	49·9	B
130	10	—	—	—	49·2	C
145	10	35·5	28·4	24·4	48·8	—
Further 35	20	(Calculated) 52·6	—	—	(47·7)	D, F

Normal serum, total protein 6·7 g./100 ml., dialyzed undiluted against 2 l. phosphate buffer (pH 8·0, $\mu = 0{\cdot}1$) for 22 hr., diluted with fresh phosphate buffer of pH 8·0, dialyzed against this buffer for 48 hr., after which the protein concentration was 2·02 g./100 ml. (Kjeldahl); electrophoresis (I) for 145 min. at 10 mA., then for 126 min. at 20 mA.; 0·3 ml. of α_2-free albumin from ascending side added to 4 ml. buffer and this solution used as supernatant on the ascending side in the experiment following (II), with the starting solution of diluted serum and buffer the same as in (I); the contents of the electrode vessels were not changed; 10 mA. corresponds to about 5·3 V./cm.; the optical enlarging factor along the direction of the current was 1·12. The composition of the starting solution as calculated from the descending pattern was: $A(+\alpha_1) = 1{\cdot}34$, $\alpha_2 = 0{\cdot}11$, $\beta = 0{\cdot}30$, $\gamma = 0{\cdot}27$ g./100 ml.; the concentration between the A and α_2 boundaries on the ascending side ($C'_{conc.}$) was approximately $1{\cdot}34 \times \rho$, i.e. 1·2; the concentration between the $A_{conc.}$ and the $A_{dil.}$ boundaries ($C'_{dil.}$) was 0·07 g./100 ml. (calculated from the areas).

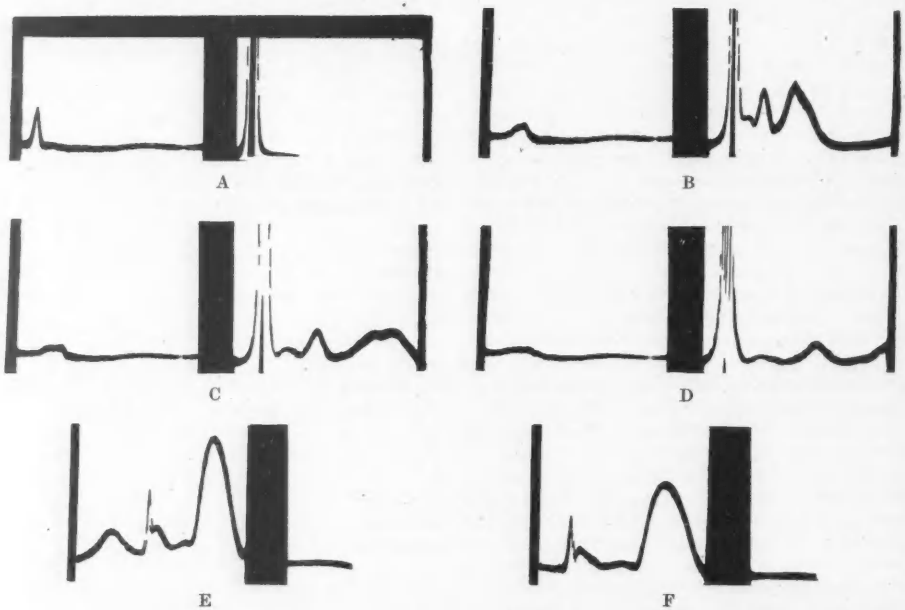

Fig. 2. Migration velocities of the boundaries of dilute and concentrated albumin. A–D, ascending patterns, tilt 45°; A, B and C after 0, 72 and 130 min. at 10 mA., D after 145 min. at 10 mA. and further 35 min. at 20 mA.; E, F, descending patterns, tilt 45°; E, after 145 min. at 10 mA.; F, after further 35 min. at 20 mA. Phosphate buffer pH 8·0, $\mu = 0{\cdot}1$.

albumin had migrated into buffer alone. Per unit distance migrated by the $A_{dil.}$ boundary, this is $\frac{rV_1K_1C'_{dil.}}{rV_1(1+K_1C'_{dil.})}$, or very nearly $K_1C'_{dil.}$. In the present case this is 0·4 % of 30·9 mm., or 0·13 mm. This figure has to be subtracted from 2·1 (the change in the distance between the two A boundaries), and the relative increment in velocity/unit concentration, K_1, becomes $\frac{1{\cdot}97}{30{\cdot}9\,(1{\cdot}2-0{\cdot}07)}$, i.e. 0·056.

The salt concentration in the layer of diluted albumin will be smaller than that which would be obtained after electrophoresis of albumin at this concentration into buffer, by the extent to which K_1 increases as the protein concentration increases. Hence a gravitationally unstable region might be expected to appear behind the $A_{dil.}$ boundary. This region would sink as soon as it was formed and would appear as an additional peak at the $A_{conc.}$ boundary. Such disturbances are vanishingly small if the concentration of the dilute albumin solution is below 0·1 g./100 ml.

It should be noted that separation into two components is seen at the boundary of the dilute but not at that of the concentrated solution. This is in accordance with the theory as will be seen in a later section of the paper. Fig. 2 D and F show the patterns after the δ and γ boundaries have reached the bridge. The appearance of the descending pattern has barely changed, since the convections were restricted to the region below the albumin boundary.

The high value for K_1, obtained when calculated from the relative positions of the α_2 and A boundaries in serum patterns, suggests a disturbance in the region of the α_2 boundary, due to inverted density gradients or a mobility ratio for the α_2-globulin across the descending albumin boundary, which deviates considerably from that assumed constant for all ions in Svensson's equation. The velocity of the α_2 boundary relative to that of the A boundary appears too large on the ascending or too small on the descending side or both. The discrepancy in the distance $A-\alpha_2$ is about 0·5 mm. on either the ascending or descending side in a normal pattern of serum diluted to a total protein concentration of 1·5 g./100 ml. (phosphate buffer pH 8·0 and $\mu=0{\cdot}1$), in which the distance between A and δ is 35 mm. The data given by Lagercrantz (1945) for the corrected mobilities of the components of normal human serum at pH 7·7 and $\mu=0{\cdot}05$ support the present findings of the anomalous position of the α_2 boundary. The values for α-globulin are 5·57 and $5{\cdot}06\times10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ for the ascending and descending sides respectively. At $\mu=0{\cdot}2$, Lagercrantz found agreement between the values for the mobilities of α-globulin.

The net charge of serum albumin. The experimental determination of K_1 makes it possible to calculate the average specific charge of serum albumin at pH 8·0. If C, the equivalent concentration of the colloid ion in Svensson's equation (23) (quoted above), is replaced by $Q_sC_1\times10^{-2}$ (Q_s being the specific charge, expressed as valence/g.) then from equation (4)

$$K_1=\frac{Q_s\times10^{-2}}{\sum\frac{c_{i1}u_{i1}}{u_{i1}-\mathbf{U}}-C}, \qquad (4a)$$

and Q_s can be calculated, C being neglected (or used as a correction term after Q_s has been found). The following numerical values were used:

$$c_{Na^+1}=-c_{HPO_4^{=}1}=0{\cdot}067\times10^{-3}\text{ equivalents/ml.,}*$$

$$u_{Na^+1}=2{\cdot}35,\dagger \quad u_{HPO_4^{=}1}=-2{\cdot}19,\ddagger$$

$\mathbf{U}=-0{\cdot}7$ (mobilities in 10^{-4} cm.^{2} V.$^{-1}$ sec.$^{-1}$),§ $K_1=0{\cdot}06$. Q_s then becomes $-2{\cdot}8\times10^{-4}$. The net charge of human serum albumin (molecular weight 70,000) at 0°, pH 8·0, and $\mu=0{\cdot}1$ is therefore about −19. Cohn, Oncley, Strong, Hughes & Armstrong (1944) quote −18 as the net charge of human serum albumin at pH 7·4. If the values for horse serum albumin B, calculated by Abramson *et al.* (1942) from a combination of electrophoresis, diffusion, and sedimentation data at 25° and $\mu=0{\cdot}1$, are extrapolated to a pH of 8·0, values of about −20 are obtained. The extrapolated values for pH 8·0 of the valence, calculated from titration data by the same authors, are about −30 and −21 for $\mu=0{\cdot}1$ and 0 respectively.

An accuracy of 10 % in the value for the net charge as determined here implies an accuracy of 1 % in the values for the boundary displacements. Both the corrections for the volume changes in the electrode vessels and those caused by changes in the solvent concentration at the boundary, which were discussed by Dole (1945), seem in this case of minor importance. One advantage of this method is its independence of the starting conditions in cases in

* A small error only is introduced by disregarding the presence of $H_2PO_4^-$ (about 10 mol./100) and assuming all phosphate to be in the form of $HPO_4^{=}$.

† Landolt-Börnstein, *Physikalisch-Chemische Tabellen.*

‡ Landolt-Börnstein, extrapolated to 0° and 0·033 mol./l. from a value given for 25° at infinite dilution, assuming the same relative dependency on temperature and concentration as the $SO_4^{=}$ ion.

§ Extrapolated from values for different ionic strengths and pH given by Lagercrantz (1945), who derived the values for the mobilities from conductivity measurements on particular sections of the contents of the **U**-tube, and others (Stenhagen, 1938; Longsworth & MacInnes, 1940*b*; Blix, 1941; Kendall, 1941; Chargaff, Ziff & Moore, 1941; Cohn *et al.* 1944; Moore, 1945).

which the Donnan equilibrium has not been completely established, since the protein concentration below the boundary can be estimated by chemical methods or from the electrophoretic pattern. But the main merit appears to be that the net charge of a leading ion can be obtained without previous separation from other components.

Boundary relationships with two components of similar mobilities

The statement of the conservation of matter applied to the protein ions overtaking an ascending boundary leads, with the assumptions made earlier in this paper, to an expression for the concentration ahead of the boundary. For the two fastest components the relationship is

$$(V_1-V_2)\,(1+K_2C_2'+K_1C_1')\,C_1' = [V_1\,(1+K_1C_1')-V_2\,(1+K_2C_2'+K_1C_1')]\,C_1',$$

where C_1', C_2' are the true concentrations behind the boundary of the slower component.

With $1+K_2C_2'+K_1C_1'=e$,

$$C_1'=\frac{1}{2K_1}\left[+\left|\sqrt{\left\{\left(1-\frac{V_2}{V_1}e\right)^2+4K_1eC_1'\left(1-\frac{V^2}{V_1}\right)\right\}}\right| -\left(1-\frac{V_2}{V_1}e\right)\right]. \quad (5)$$

With $C_2'/C_1'=n$, $K_2/K_1=p$, and $V_1/V_2=e$ (i.e. the percentage difference in the mobilities being the same as the percentage increment in the velocity due to the protein concentration behind the boundary), $C_1'=C_1'\sqrt{(pn+1)}$.

The separation S of the boundaries is given by

$$S=\frac{C_1'}{C_1'}(V_1-V_2)\,(1+K_1C_1'+K_2C_2'). \quad (6)$$

A limiting case of interest arises when $p=1$ and V_1 nearly equal to V_2; the faster component will adjust itself ahead of the boundary to a concentration nearly equal to the total concentration behind the boundary, independent of the relative concentrations in the mixture. When the mobilities are similar and in addition $p>1$, a gravitationally unstable region may be formed ahead of the boundary of the slower component.

It can be seen by inspection of equation (5) that, by accumulation, the faster component can increase in concentration ahead of a boundary to a multiple of that behind the boundary. It can thus reverse the ratio of the apparent concentrations of two components under conditions normally adhered to, such as, for example, a concentration of proteins of 1 g./100 ml. if the mobilities of the components differ by as much as 5%. The following example illustrates this. Fig. 3 shows the A_1, A_2 (Blix, Tiselius & Svensson, 1941; Hoch & Morris, 1945) and α_2 boundaries after an electrophoresis at pH 8·0, and the αA, βA (Luetscher, 1939), and a small component (possibly α_1) after an experiment at pH 4·0. The apparent concentration of A_1, the fastest component at pH 8·0, is higher than that of A_2. At pH 4·0, where the serum components migrate in the reverse order and where the serum albumin shows two components (αA and βA) having a much greater difference in mobility than that of the two components at pH 8·0, it is also the faster component (αA) which appears higher in concentration. As a consequence of equation (5) these observations suggest that the two components at pH 8·0 were the same as those at pH 4·0 and not that they were

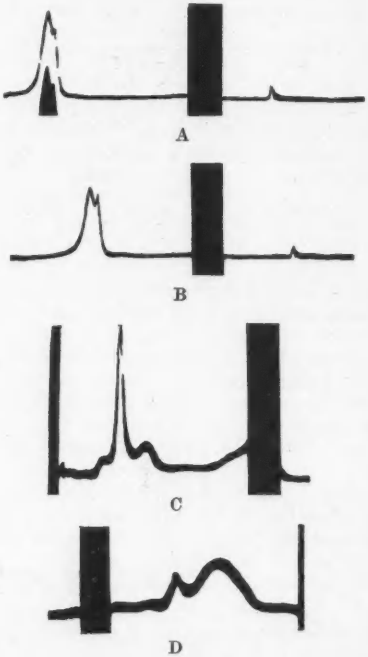

Fig. 3. Apparent proportions of the components of serum albumin. A, B, ascending patterns, showing albumin and α_2 boundaries after prolonged electrophoretic runs on serum. Protein concentration 2·4 g./100 ml.; phosphate buffer pH 8·0, $\mu=0{\cdot}1$. Comparison of B with A shows the reproducibility after the serum had been kept for 16 days at pH 8 in the refrigerator. A, after 550 min. at about 19·5 mA., tilt 67° to the horizontal; B, after 540 min. at 19–19·5 mA., tilt 70°. C ascending and D descending patterns of serum albumin free from α_2, obtained from experiment of Fig. 3B; tilt 25° to the horizontal; protein concentration 0·29 g./100 ml.; acetate buffer pH 4, $\mu=0{\cdot}02$.

different, as might appear from their relative areas, although they may be so for other reasons.

The degree of separation for a particular case of a 2 : 3 mixture of two proteins was calculated for two total concentrations of 1·2 and 0·1 g./100 ml. protein. The velocities at infinite dilution were taken to differ by 7 % ($V_1/V_2 = 1{\cdot}07$). The degree of separation for the higher concentration (with $K_1 = K_2 = 0{\cdot}06$, $p = 1$, and $\mathbf{C}_1' = 0{\cdot}48$) works out to 1/1·58 times the separation at infinite dilution. For the lower concentration ($\mathbf{C}_1' = 0{\cdot}04$), the degree of separation is very nearly equal to (1/1·04 times) the separation at infinite dilution. If the latter were about 2 mm. one would expect a separation of 1·9 mm. at 0·1 g./100 ml. protein and a separation of 1·27 mm. at 1·2 g./100 ml. protein.

The relationship between concentrations of a protein component ahead and behind a descending protein boundary is given by

$$C_2 = \frac{1}{2K_2}\left[-\left|\sqrt{\left\{\left(\frac{V_1}{V_2}g-1\right)^2 - 4K_2 g\mathbf{C}_2\left(\frac{V_1}{V_2}-1\right)\right\}}\right| + \left(\frac{V_1}{V_2}g-1\right)\right], \quad (7)$$

where $g = 1 + K_1\mathbf{C}_1 + K_2\mathbf{C}_2$; $\mathbf{C}_1$, $\mathbf{C}_2$ are the true concentrations of the components ahead of the boundary of the faster component.

The extent to which analyses of serum patterns may give values for concentrations that are different from those of the original mixture can be assessed

Table 4. *Apparent proportions of the faster component of a two-component mixture, calculated from equations* (5) *and* (7)

	Total concentration (g./100 ml.)						Difference between means
	0·6			2·4			
($V_2/V_1 = 0{\cdot}4$)	Asc.	Desc.	Mean	Asc.	Desc.	Mean	(%)
	Faster component 0·725 of total						
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}06$	0·736	0·730	0·733	0·759	0·741	0·750	2·3
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}03$	0·731	0·730	0·731	0·742	0·741	0·742	1·4
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}09$	0·742	0·730	0·736	0·776	0·741	0·759	3·0
	Faster component 0·5 of total						
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}06$	0·516	0·506	0·511	0·545	0·521	0·533	4·2
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}03$	0·507	0·506	0·507	0·523	0·521	0·522	3·1
$K_1 = 0{\cdot}06$ $K_2 = 0{\cdot}09$	0·521	0·506	0·514	0·570	0·521	0·545	5·1

The differences between the values for the total concentrations of 0·6 and 2·4 g./100 ml. are here slightly underestimated since the dependency of K_f on the protein concentration has not been taken into account. The numerical evaluation of equation (4*a*) suggests that a value for K_1 of 0·06 should be increased by about 7 % when the protein concentration rises by 1 g./100 ml.

An example for such conditions might be represented by Fig. 2C. The separation of the two components at the small-albumin boundary is between 2 and 3 mm. At the large-albumin boundary no split could be observed, but a difference in the width of the boundary of about 0·7 mm. was found between patterns B and C (measured at 10–15 mm. above the base line). This corresponds to a spreading of about 1·4 mm. during the experiment between patterns A and C.

In view of the fact that other components with mobilities similar to that of the albumin, apart from A_1 and A_2, may possibly be present in small quantities, the spreading of the large albumin boundary seems in fair agreement with the theory.

from Table 4, which has been computed from equations (5) and (7) for a two-component system simulating one containing an albumin and a globulin component, of mobilities in the ratio 1 : 0·4. The calculation was carried out for different values of K_2 and for two relative proportions of the two components, one being similar to that given in Table 2 for $(A + \alpha_1 + \alpha_2) : (\beta + \gamma)$, i.e. the faster component 72·5 % of the total. The concentrations on the ascending side were calculated from those assumed for the original mixture by multiplication by ρ. Although ρ is dependent on K_2, K_1 being kept constant, a change in ρ of 5 or 10 % affects the values given in Table 4 only slightly, and therefore ρ was kept the same for all values of K_2. The values used,

0·97 and 0·87 for the lower and higher concentrations respectively, were average experimental values found for serum.

SUMMARY

1. The anomalies in electrophoretic patterns of protein mixtures are considered theoretically for specified conditions, and the equations derived are applied to the study of normal and pathological sera.

2. The value for a coefficient which relates the change in migration velocity of protein ions to the change in protein concentration was determined experimentally for human serum albumin. It was found to be $K_1 = 0{\cdot}06$, signifying an increase of 6 % in the migration velocity of an ascending boundary/g. protein/100 ml.

3. The average net charge of the albumin was calculated by inserting this value into an expression derived from the equation for the conductivity change across a colloid boundary given by Svensson (1946). A value for the valence of about -19 was found, which is consistent with that of -18, quoted by Cohn *et al.* (1944) for pH 7·4, obtained from measurements of electromotive force.

4. The α-globulin boundary in patterns of serum in phosphate buffer at pH 8·0 and $\mu = 0{\cdot}1$ was found to have a slightly anomalous position relative to that of the albumin boundary.

5. A method for comparing mobilities by simultaneous measurements is outlined.

6. The calculation of the concentration relationships in a boundary region of two components reveals that the relative concentrations as deduced from the apparent concentration changes at the boundaries give a grossly erroneous picture of the concentrations in the original solution, when the value for the relative difference in the mobilities is in the region of that of K_1 or lower.

7. The degree of separation of two boundaries depends on the total concentration. The examination for electrophoretic homogeneity should be carried out at low concentrations where the separation of the boundaries is greatest and over a range of concentrations as far as the interference of diffusion allows. An experiment on human serum is described in which separation of the albumin boundary into two components was observed at a concentration of 0·07 % after a migration of 31 mm., while no separation but only broadening was seen at a concentration of 1·2 %.

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While this paper was being prepared for press, a publication by Longsworth (1947) appeared dealing with related problems.

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The Use of Phenol Glucuronide in the Assay of β-Glucuronidase

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Our present knowledge of the distribution in the body and the physical properties of β-glucuronidase is derived almost entirely from experiments, in which free glucuronic acid was determined by reduction of ferricyanide after enzymic hydrolysis of a conjugated glucuronide. Whilst it is possible to determine very small amounts of glucuronic acid by means of its reducing power (Levvy, 1946), the lack of specificity in the reaction is a serious disadvantage in the routine assay of glucuronidase. Unless the enzyme preparation has been considerably purified, it may contain large amounts of materials other than glucuronic acid which reduce ferricyanide (Levvy, 1948). Other impurities may interfere during the actual titration, if this is cerimetric, obscuring the end point and making it unstable. In studying the enzymic hydrolysis of oestriol glucuronide, Fishman (1939) determined free oestriol by the Kober colour reaction. Its scarcity, however, renders this substrate unsuitable for routine assays. Talalay, Fishman & Huggins (1946) have recently described a method whereby the hydrolysis of phenolphthalein glucuronide can be followed colorimetrically. This method appears to avoid the difficulties mentioned above in the determination of glucuronidase activities. It was found possible in this laboratory to apply the method of King & Armstrong (1934) for phosphatase determination to the assay of glucuronidase, using biosynthetic phenol glucuronide (Williams, private communication) as substrate. (We are greatly indebted to Dr Williams for details of the technique for obtaining this compound from rabbit urine.) Results obtained in studying the kinetics of hydrolysis of phenol glucuronide with enzyme obtained from two different organs, and a description of the procedure for the routine assay of glucuronidase in mouse tissues, are given below.

EXPERIMENTAL

Preparation of phenol β-D-glucuronide. The glucuronide was obtained from the urine of rabbits fed with phenol by the general procedure described by Williams (1943) for the isolation of aminophenol glucuronides. Before use, it was dried over P_2O_5 *in vacuo* for 10 hr. at 80°. Analysis showed it to be free from solvent of crystallization (m.p. 162°, corr.).

The colour reaction for free phenol. Phenol was determined with the reagent of Folin & Ciocalteu (1927). King & Armstrong (1934) give full details of the technique for this estimation. The colour intensity was measured with a Spekker photoelectric absorptiometer, using Ilford no. 602 blue filter. Free glucuronic acid did not interfere in the reaction. Phenol glucuronide in high concentration gave a faint colour, probably due to traces of free phenol (less than 0·2% of the total weight), since it varied from one sample to another. Contrary to the findings of Folley & Kay (1935), the reaction was not particularly sensitive to the Na_2CO_3 concentration and was complete in less than 20 min. at 37°. In constructing the calibration curve (Fig. 1),

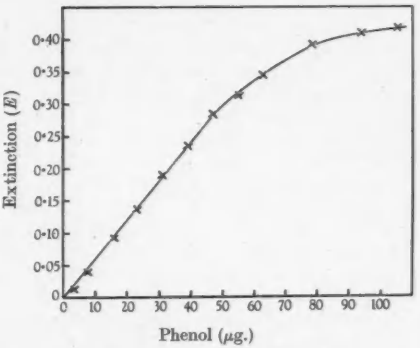

Fig. 1. Calibration curve for estimation of phenol.

0·6 ml. standard phenol solution (Hawk & Bergeim, 1931) was added to 0·2 ml. 0·1 M-citrate buffer (pH 5·2), followed by 2 ml. of a 1 : 5 dilution of the Folin-Ciocalteu reagent. After mixing, 2 ml. of the liquid were taken for colour development as described below for enzyme extracts. Citrate, acetate and formate buffers had no effect on the colour reaction.

Preparation of the enzyme. Preparations from mouse liver and spleen were studied separately in these experiments. The organs were broken up in glass homogenizers (Umbreit, Burris & Stauffer, 1945). Unless stated to the contrary, the enzyme was partially purified following the method described by Talalay *et al.* (1946). The homogenate was brought to pH 5·0 or 5·2 with acetic or citric acid. Protein which separated after 30 min. at 37° was removed by centrifuging, and the enzyme in the supernatant liquid precipitated by adding an equal volume of saturated

$(NH_4)_2SO_4$ solution (SAS). The precipitate was dissolved in a convenient volume of water. No change in the amount of enzyme present was noted after purification in this way.

The effect of pH. The pH for optimum hydrolysis was determined over the range 3–6·5 at a substrate concentration of 0·01 M in citrate buffer. To 0·2 ml. of 0·1 M-buffer and 0·2 ml. of 0·04 M-substrate warmed to 37° was added 0·4 ml. enzyme, and the mixture was incubated at 37° for 1 hr. The initial effect of substrate and enzyme on the pH of the buffer was determined electrometrically. No further change in the pH occurred during the incubation.

With crude homogenates of liver and spleen, considerable amounts of substances giving the colour reaction were liberated in absence of substrate. It would appear that an enzymic process was responsible for the production of these compounds since the amount formed varied with the pH, with an optimum at 3·6–4·6. Partial purification of the homogenates as described above made the blank much smaller and very nearly independent of the pH. With purified enzyme from liver and spleen, the curves for variation in hydrolysis with pH assumed the shapes shown in Fig. 2. Homogenates from five mice were pooled for each organ before purifying, and all points on the curve were determined simultaneously. Corrections were applied for the enzyme blank at the appropriate pH (corresponding to about 200 μg. phenol/g. for spleen and about 100 μg./g. for liver) before plotting the points. From Fig. 2 it can be seen that for enzyme from both sources the rate of hydrolysis rose to a maximum at pH 5·2 as the pH was lowered. After a drop at pH 4·75, the velocity again rose slightly at pH 4·5 and then fell off rapidly. The subsidiary peak at pH 4·5 was seen in other experiments when the pH increment was made small enough. A possible explanation of the shape of the curves in Fig. 2 is given by the work of Mills (1947), who found that β-glucuronidase from beef spleen could be separated into two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide. In acetate buffer, some results were obtained which suggested the pH optimum to be lower than in citrate, but this point was not further investigated. It was decided that for the routine assay of the enzyme the hydrolysis of phenol glucuronide should be done at pH 5·2 in citrate buffer.

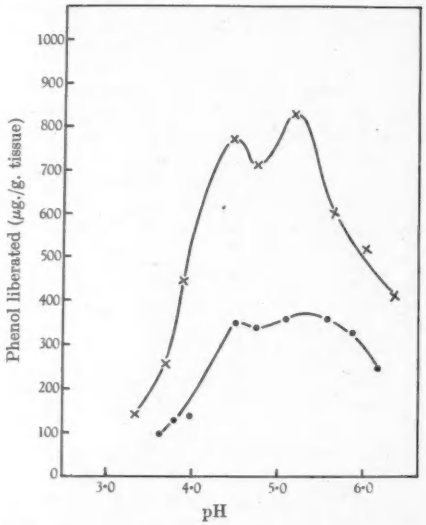

Fig. 2. The variation in hydrolysis rate of phenol β-D-glucuronide with pH. Results expressed in terms of phenol liberated/g. moist tissue. ×—× spleen enzyme; •—• liver enzyme.

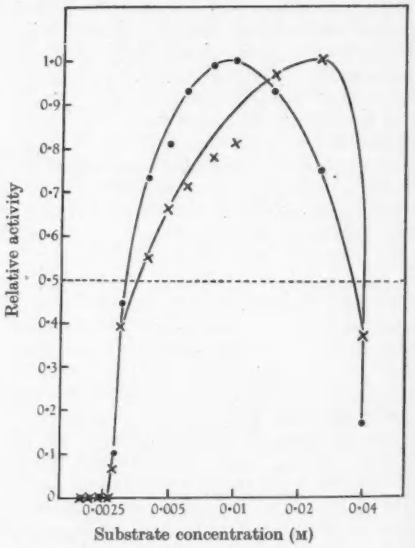

Fig. 3. The variation in initial hydrolysis rate with substrate concentration. Results expressed as fraction of maximum observed activity. ×—× spleen enzyme; •—• liver enzyme.

The effect of substrate concentration. The effect of varying the substrate concentration at constant pH is shown in Fig. 3. The citrate buffer and substrate solutions were adjusted to pH 5·2. Addition of enzyme caused no appreciable change in pH. Three experiments were done with enzyme from each source and the averaged results are shown in the figure. It will be noted that inhibition by excess

substrate was marked in both cases. The substrate optimum for spleen (0·025M) was greater than for liver (0·01M). The results did not lend themselves to the accurate calculation of the Michaelis-Menten constant, K_m, by the method of Lineweaver & Burk (1934), but the value obtained graphically from Fig. 3 by finding the substrate concentration at

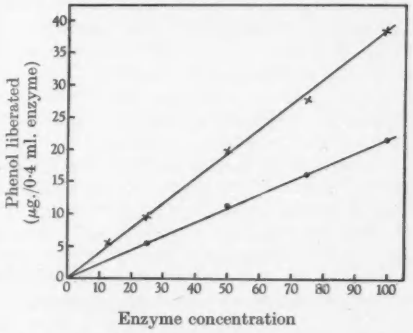

Fig. 4. The relation between enzyme concentration and phenol liberated under standard conditions. Enzyme concentration in arbitrary units. ×—× spleen enzyme; •—• liver enzyme.

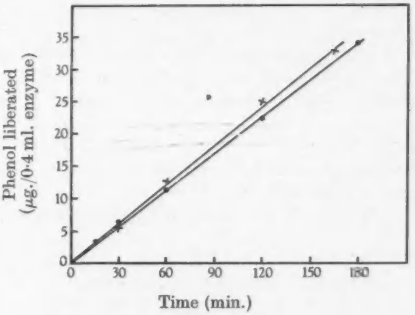

Fig. 5. Phenol liberated after varying incubation periods. ×—× spleen enzyme; •—• liver enzyme.

which half the observed maximum velocity of hydrolysis was developed was approximately the same (0·0035M) for enzyme from either source. The difference in shape between the curves for liver and spleen may be due to the presence of interfering substances in the tissue extracts rather than a qualitative difference in the enzyme system. A substrate concentration of 0·015M was adopted for further work with enzyme from both sources.

The effect of varying the enzyme concentration and the duration of hydrolysis. As shown in Fig. 4, the amount of phenol liberated in 1 hr. at 37° and pH 5·2 (citrate buffer) from 0·015M-phenol glucuronide was directly proportional to the enzyme concentration. Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 3 hr. (Fig. 5).

The assay of β-glucuronidase

Reagents

Citrate buffer. (*a*) 0·3M, pH 5·2 (glass electrode); (*b*) 0·1M, prepared from 0·3M.

Substrate solution. 0·06M-Phenol glucuronide adjusted to pH 5·2; stored in the refrigerator.

Folin-Ciocalteu reagent. (*a*) Stock solution (British Drug Houses Ltd.); (*b*) Stock solution diluted 1 to 5 and prepared daily.

N-Sodium carbonate solution.

Saturated ammonium sulphate solution. Adjusted to pH 5·2.

Procedure

Spleen. The dissected organ was weighed in a chilled homogenizer tube and homogenized with 3 ml. water. The cell-free homogenate was transferred to a graduated centrifuge tube, using 1 ml. wash water. After addition of 0·5 ml. 0·3M-buffer, the tube was maintained at 37° for 30 min. Coagulated protein was separated by centrifuging for 15 min. The volumes of precipitate and supernatant liquid were noted and the latter was transferred to another graduated centrifuge tube. An equal volume of SAS was added and the tube centrifuged for 30 min. The liquid was discarded, and the residue dissolved in water and made up to a volume of 2 ml. The concentration of enzyme was then such that, in the case of normal animals, 0·4 ml. of the solution gave readings of 20–40 μg. phenol after corrections had been applied for enzyme and substrate blanks. This volume of the enzyme solution was added to 0·2 ml. of 0·1M-buffer and 0·2 ml. substrate in a stoppered 10 ml. centrifuge tube. Prior to addition of the enzyme, the substrate and buffer were warmed to 37°. After incubation of the hydrolysis mixture for 1 hr. at 37°, 2 ml. of the diluted Folin-Ciocalteu reagent were measured into the tube. Protein was removed by centrifuging for 3 min. and 2 ml. of the supernatant transferred to a 10 ml. stoppered centrifuge tube containing 4 ml. Na_2CO_3 solution. The contents of the tube were mixed and the colour developed by incubating for 20 min. at 37°. Assays were carried out in duplicate.

Liver. The procedure was exactly as described above except that at certain stages the volumes used differed. Homogenization was done with 5 ml. water and 3 ml. of water were added in transferring the homogenate to the first graduated tube. In adjusting the pH, 1 ml. 0·3M-buffer was used. The solution of the enzyme after SAS precipitation was

made up to 4 ml., this volume giving readings of 20–40 μg. phenol in the assay after correction for blanks with livers from normal animals.

Controls. Every assay was controlled by incubating mixtures of enzyme and buffer containing 0·2 ml. water in place of substrate. The purified enzyme shows a small rise in the blank during incubation. Controls for phenol in the substrate were also done at frequent intervals, 0·2 ml. of the solution being incubated with 0·2 ml. buffer and 0·4 ml. water. Since there appeared to be a very small but perceptible increase in the reading when the substrate was incubated at pH 5·2, this procedure was considered preferable to that in which substrate is added to the enzyme after incubation (cf. Talalay *et al.* 1946).

RESULTS

The recoveries obtained in a series of experiments in which 0·2 ml. standard phenol solution was added to 0·2 ml. 0·1 M-buffer and 0·4 ml. enzyme (spleen and liver mixed) are shown in Table 1. In some experiments the mixture was incubated whilst in others the Folin-Ciocalteu reagent was added at once. Each figure shown in the table is the result of a determination carried out in duplicate and the results have been corrected for the enzyme blank (5·2 μg. unincubated, 7·7 μg. incubated).

Table 1. *Recovery of phenol added to buffered enzyme*

Phenol added	Phenol recovered before incubation		Phenol recovered after incubation	
(μg.)	(μg.)	(%)	(μg.)	(%)
3·9	3·7	95	4·1	105
7·9	8·0	101	8·0	101
15·7	15·6	99	15·8	101
31·4	31·1	99	31·2	99
62·9	63·1	100	63·0	100
105·0	105·0	100	104·9	100

In a series of 30 determinations in which 31·4 μg. phenol were added to enzyme and buffer as above, and determined at once, the mean recovery was 31·5 μg. (100·3 %), and the standard deviation of a single determination from the mean was 0·19 μg. (0·6 %).

DISCUSSION

From the results given above it can be seen that phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the reagent of Folin & Ciocalteu for estimation of phenol liberated by the enzyme. As compared with phenolphthalein glucuronide (Talalay *et al.* 1946), a much higher concentration of phenol glucuronide is required for optimum hydrolysis, and inhibition of the enzyme by excess substrate is more pronounced.

It has been generally assumed without any experimental justification that the conditions for optimum hydrolysis by β-glucuronidase are always the same no matter the source of the enzyme. The data obtained in this work for mouse liver and spleen glucuronidase support the assumption. Masamune (1934), in his pioneer work on glucuronidase, found hydrolysis of phenol glucuronide by beef kidney extracts, determined by estimation of glucuronic acid, to be at a maximum at pH 5·3–5·6 in citrate buffer. This is in good agreement with our own figure of 5·2 for mouse liver and spleen extracts.

SUMMARY

1. Phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the Folin & Ciocalteu (1927) reagent for the estimation of free phenol.

2. In the procedure adopted for assay of glucuronidase in mouse liver and spleen, hydrolysis of phenol glucuronide was carried out in citrate buffer at pH 5·2 and a substrate concentration of 0·015 M.

3. The mean recovery of added phenol in the assay procedure was 100 %, and the standard deviation of a single observation from the mean was 0·6 %.

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Studies in Vitamin A

4. SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN A IN LIVER OILS. CORRECTION FOR IRRELEVANT ABSORPTION

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The ultraviolet absorption spectrum of vitamin A with its maximum near 328 mμ. was described nearly 20 years ago (Morton & Heilbron, 1928*a*, *b*). The intensity of absorption shown at that wave length by a fish-liver oil or concentrate was conveniently expressed in terms of $E^{1\%}_{1\,cm.}$ 328 mμ. (Gillam & Morton, 1929), and this magnitude was early seen to afford a guide to potency. If a number of fish-liver oils covering a fair range of potency was examined in three different ways, namely, (*a*) by the method of biological assay, (*b*) by the antimony trichloride colour test, and (*c*) by the spectrophotometric method, the results showed fair agreement. From a strictly quantitative standpoint the bio-assays were primitive, the colour test was handicapped by a highly variable degree of inhibition and the ultraviolet absorption by superimposed irrelevant absorption.

Experience gained by a very large number of workers has since shown that bio-assays cannot compete with the spectrophotometric method in terms of either convenience or accuracy. In fact, for a bio-assay to be worth doing to-day a very large number of animals needs to be used, and the data must be subjected to rigorous statistical study. This makes it all the more necessary to dispose of difficulties and complications in the spectroscopic procedure.

The broader aspects of the problem of vitamin A activity raise acute difficulties (Morton, 1942; Hickman, 1943; Gridgeman, 1944), and it is proposed at this stage to confine attention mainly to the optimal use of spectrophotometry. We now enjoy advantages which were previously lacking. Vitamin A and its acetate are now available as pure, crystalline substances, and photoelectric spectrophotometry is capable of a higher degree of accuracy than photographic methods. Irrelevant absorption can be allowed for (Morton & Stubbs, 1946) so that the true intensity of absorption attributable to the vitamin A can be determined. By the use of such a correction procedure one major difficulty has been removed. This difficulty concerned the relationship between $E^{1\%}_{1\,cm.}$ 328 mμ. and potency determined by large-scale co-operative feeding trials with (*a*) halibut-liver oil and (*b*) crystalline vitamin A β-naphthoate. The conversion factors differed significantly, but the discrepancy disappeared when the gross intensity of absorption shown by the halibut-liver oil was suitably corrected for irrelevant absorption (Morton & Stubbs, 1947).

The purely analytical problem of determining the vitamin A content of an oil does not, however, of necessity require any reference to conversion factors or to the international unit of vitamin A activity (0·6 μg. of β-carotene). From many points of view further studies on the wider problem would be made easier if 'potencies' could be replaced by 'vitamin content' expressed in mg./g.

Spectrophotometric characteristics of vitamin A (free alcohol) and vitamin A acetate

Any attempt to determine vitamin A in an oil (where it normally occurs mainly as esters), or in an unsaponifiable fraction, requires at least accurate values for the molecular extinction coefficient (ϵ) or $E^{1\%}_{1\,cm.}$ at the maximum for the free or esterified vitamin dissolved in a specified solvent. For the extended use of spectrophotometry the whole absorption curve needs to be known somewhat accurately so that irrelevant absorption can be estimated.

Crystalline vitamin A acetate dissolved in *cyclo*hexane shows a maximum at 328 mμ. The intensities of absorption at different wave lengths are shown in Table 1 and are expressed as fractions of the peak value. The two wave lengths, one on each side of the maximum, at which the intensity of absorption is exactly 6/7 of the peak value have been determined as carefully as possible as 313 and 338·5 mμ. The same wave lengths apply to the purest natural ester preparations (Morton & Stubbs, 1946). It is to be noted that these figures apply to *cyclo*hexane solutions, because the absorption spectra of vitamin A and its esters vary a little from solvent to solvent. The absorption curve for the free vitamin differs a little from that of the esterified vitamin. Small though the difference is, it must be taken into account in applying correction procedures. Table 1 contains enough information for most purposes. The only significance of the ratio 6/7 is that it is empirically appropriate in relation to the wave length range covered and to the performance of the spectrophotometer.

Table 1. *Intensities of absorption of vitamin A and vitamin A acetate in different solvents, expressed as a fraction of $E_{max.}$ for wave lengths between 220 and 450 mμ.*

λ (mμ.)	Crystalline vitamin A alcohol		Crystalline vitamin A acetate		
	*cyclo*Hexane	Ethanol	*cyclo*Hexane	Ethanol	75% (v/v) aqueous ethanol
220	0·143	0·089	—	0·106	—
230	0·127	0·093	—	0·106	—
240	0·128	0·102	—	0·113	—
245	—	0·107	—	—	—
250	0·125	0·112	0·135	0·123	0·123
255	0·126	0·112	0·137	—	—
260	0·123	0·109	0·136	0·122	0·133
265	0·127	0·115	0·138	—	—
270	0·150	0·139	0·158	0·150	—
275	0·187	0·175	0·193	—	—
280	0·236	0·224	0·238	0·254	0·265
285	0·300	0·313	0·300	0·337	—
290	0·393	0·414	0·373	0·410	0·405
295	0·515	0·518	0·469	0·514	—
300	0·611	0·609	0·571	0·604	0·592
305	0·718	0·720	0·668	0·716	—
310	0·841	0·844	0·795	0·826	0·814
311	0·861	0·857	—	0·845	—
311·5	—	—	—	0·854	0·840
312	0·877	—	0·838	0·864	0·844
312·5	—	—	—	—	0·858
313	0·888	—	0·858	0·878	0·863
315	0·906	0·898	0·889	0·897	0·888
317·5	0·923	0·922	0·915	0·927	—
320	0·950	0·950	0·934	0·953	0·946
322·5	0·978	0·983	0·961	—	—
325	0·998	1·000	0·988	0·999	0·991
326	1·000	1·000	—	1·000	0·999
326·5	—	—	—	—	1·000
327	0·998	0·990	—	1·000	0·999
328	0·994	—	1·000	0·990	0·991
330	0·972	0·957	0·995	0·976	0·982
335	0·880	0·869	0·925	0·905	0·914
335·5	—	0·861	—	—	—
336	0·861	0·849	—	0·886	—
337	—	—	—	0·868	—
337·5	—	—	—	0·860	0·872
338	—	—	—	0·852	—
338·5	0·810	—	0·857	—	0·855
340	0·776	0·772	0·828	0·812	0·827
345	0·646	0·638	0·707	0·689	—
350	0·513	0·509	0·572	0·557	0·580
355	—	0·397	0·440	0·437	—
360	0·293	0·278	0·331	0·317	0·359
365	0·207	0·172	0·239	—	—
370	0·129	0·108	0·151	0·132	0·175
375	—	0·074	—	—	—
380	0·046	0·048	—	0·043	—
390	0·025	0·024	—	0·013	—
400	0·012	0·013	—	0·004	—
450	0·0003	0·0018	—	0·0002	—
	$\lambda_{max.}$ 326 mμ.	$\lambda_{max.}$ 325 mμ.	$\lambda_{max.}$ 328 mμ.	$\lambda_{max.}$ 326·5 mμ.	$\lambda_{max.}$ 326·5 mμ.
	$\epsilon_{max.}$ 48,310	$\epsilon_{max.}$ 51,100	$\epsilon_{max.}$ 48,460	$\epsilon_{max.}$ 50,020	$\epsilon_{max.}$ 50,510
	$E_{311}=E_{336}$	$E_{311}=E_{335·5}$	$E_{313}=E_{338·5}$	$E_{311·5}=E_{337·5}$	$E_{312·5}=E_{338·5}$
	$\frac{E_{326}}{E_{311}}=7/6$	$\frac{E_{325}}{E_{311}}=7/6$	$\frac{E_{328}}{E_{313}}=7/6$	$\frac{E_{326·5}}{E_{311·5}}=7/6$	$\frac{E_{326·5}}{E_{312·5}}=7/6$

The need for correction of data on liver oils

For many years it has been the custom to prepare the unsaponifiable fractions of low-potency oils prior to making spectrophotometric readings. It is not necessary to weigh the unsaponifiable fraction, as the intensity of absorption is more conveniently expressed in terms of the $E^{1\%}_{1\,cm.}$ value calculated on the weight of oil used. As compared with readings made on unsaponified oils, $E_{max.}$ is significantly reduced in low-potency oils, and the whole absorption curve approximates in shape to that shown by high-potency oils and rich concentrates. It was not unreasonable to assume that substances giving rise to irrelevant absorption and rendered water soluble by saponification would occur to roughly the same extent in all fish-liver oils. If that were the case such irrelevant absorption would constitute a negligible fraction of the total absorption at 328 mμ. in rich oils. Support for the assumption was provided by the observation that practically all the absorbing material of rich oils remained in the unsaponifiable fraction.

The advantage of the data in Table 1 is that the presence of irrelevant absorption becomes easy to detect. If the sample under test, e.g. a fish-liver oil, is free from irrelevant absorption the line joining the 313 and 338·5 mμ. points on its absorption curve will be horizontal at a level 6/7 of the intensity at 328 mμ. (esterified vitamin A in *cyclo*hexane). This condition is rarely satisfied, and some correction is needed even with high-potency oils. A good example is provided by the halibut-liver oils assayed carefully in 1936 and retested spectrophotometrically in 1946 (Morton & Stubbs, 1947).

The reason why irrelevant absorption occurs so frequently in high-potency oils is that vitamin A decomposition products are formed either before or during the process of extracting livers. There may also be deterioration during transit or storage. Whatever the exact reason for their occurrence, the fact of contamination by such artifacts is undeniable. Most of the decomposition products remain in the unsaponifiable fraction.

Correction procedure

The simple correction procedure described by Morton & Stubbs (1946) rests upon the assumption that the irrelevant absorption 'curve' is linear over the approximate range 310–340 mμ., i.e. that no impurity or artefact shows a maximum very close to that of vitamin A. A good deal is now known concerning the spectra of vitamin A_2 and oxidation products of vitamin A, and it can be said that over the range 310–340 mμ. their absorption curves are at least approximately linear. Nevertheless, slight departures from linearity could give rise to appreciable errors in the final result, and the final verdict on the validity of the assumption must rest on the kind of experience recorded below.

The three chosen wave lengths, $\lambda_{max.}$ for vitamin A alcohol or ester in a specified solvent and the two at which the E value is 6/7 of $E_{max.}$, may be called for convenience 'fixation points'. With a known concentration of oil or concentrate (in the specified solvent), such that $E_{max.}$ for a 1 cm. cell occurs in the region of optimal performance for the photoelectric spectrophotometer, E values are determined at the three fixation points. Normally the values on either side of the maximum are different and both exceed 6/7 of the peak value. The correction is made in two steps as in the example given below:

Cod-liver oil, 1 % solution in *cyclo*hexane:

mμ.	E	Δ mμ.	
313	0·640	15	25·5
328	0·712	10·5	
338·5	0·620		

(i) $E_{313\,m\mu.} - E_{338{\cdot}5\,m\mu.} = 0{\cdot}020$:

By similar triangles the correction for slope is

$$\frac{0{\cdot}020 \times 10{\cdot}5}{25{\cdot}5} = 0{\cdot}008,$$

$$0{\cdot}712 - 0{\cdot}008 = 0{\cdot}704.$$

(ii) Correction for the height of the general absorption:

$$\frac{0{\cdot}704 - x}{0{\cdot}620 - x} = \frac{7}{6}, \quad x = 0{\cdot}116,$$

$$0{\cdot}704 - 0{\cdot}116 = 0{\cdot}588,$$

$E^{1\%}_{1\,cm.}$ 328 mμ. (0·712 gross) = 0·588 (corrected).

Given this one point, i.e. E 328 mμ., corrected, the whole corrected vitamin A absorption curve can be constructed from the data in Table 1. The difference between this curve and the uncorrected curve will represent the spectrum of substances other than vitamin A, provided that the assumption of linearity over the fixation range is valid.

It will be convenient first to consider tests carried out on liver oils from the tunny (*Thunnus thynnus*). Portuguese workers (Gouveia, Coelho, Gouveia & Paz, 1943), in confirmation of other experience, have recorded high figures for tunny-liver oils. In some fishes, livers weighing more than 1 kg. yielded 10 % of oil, some 40 % of which seemed to consist of vitamin A esters. The absorption curves of commercial tunny-liver oils now being imported into this country appear at first sight 'normal' enough to need little correction, but when judged by the criteria of Table 1 substantial correction is seen to be essential. Granted that the uncorrected curve fails to satisfy the fixation criteria it is desirable to

provide independent checks on the correction procedure, and so indirectly to test the validity of the assumption that irrelevant absorption is linear over the fixation range.

If the correction procedure is found to withstand successfully the most searching tests which can be devised, it will then be of interest to ascertain the magnitude of the correction appropriate to some of the principal types of fish-liver oils and concentrates.

The next step will be to subject the correction procedure to the severe test of indicating correctly the proportion of vitamin A_2 in liver oils which contain both vitamin A and vitamin A_2.

The most severe test of all will be to apply the procedure to whale-liver oils, first those of high quality, i.e. refined oils in which the main absorbing constituent other than vitamin A is kitol, and secondly to crude oils in which the gross absorption is several times greater than that properly attributable to vitamin A. If the method can bear this weight of interpretation the purely analytical problem will have been solved and the way cleared for studies concerned with utilization of vitamin A in the body.

RESULTS

The Beckman ultraviolet photoelectric spectrophotometer has been used throughout.

Spectroscopic data on high-potency tunny-liver oils

Sample A

(i) Solution in *cyclo*hexane, 0·001 %; $\lambda_{max.}$ occurred at 328 mμ., $E^{1\%}_{1\,cm.}$ 328 mμ., 482 (gross); inflexions near 370 and 392 mμ., indicative of 'cyclized' or anhydro-vitamin A, were observed. $E^{1\%}_{1\,cm.}$ 328 mμ., 400 (corrected on the basis of esterified vitamin A). The subtraction curve was almost horizontal at 310–340 mμ., but irrelevant absorption was considerable, because of the presence both of 'cyclized' vitamin A and oxidation products (Fig. 1).

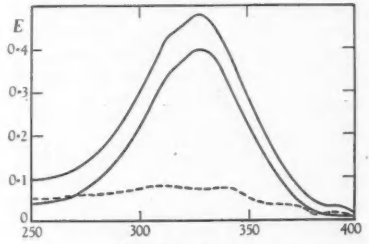

Fig. 1. Absorption curve for high-potency tunny-liver oil; upper curve observed on 0·001 % solution in *cyclo*hexane; lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(ii) A portion of oil (0·5 g.) was saponified; yield of unsaponifiable fraction 56 %, $E^{1\%}_{1\,cm.}$ 326 mμ., 802; $SbCl_3$ colour test: $E^{1\%}_{1\,cm.}$ 693 mμ., 168; 618 mμ., 2300; 582 mμ., 1105; some vitamin A_2 was present because the 693 mμ. maximum was seen clearly. $E^{1\%}_{1\,cm.}$ 326 mμ. (corrected on basis of free vitamin A) 637; recalculated on weight of oil taken, 357.

(iii) A portion of oil (0·5 g.) was saponified in the presence of a little quinol as anti-oxidant; yield of unsaponifiable matter 57·2 %; $\lambda_{max.}$ occurred at 326 mμ. (*cyclo*hexane); $E^{1\%}_{1\,cm.}$ 326 mμ., 791, or 452·5 calculated on oil; $SbCl_3$ test $E^{1\%}_{1\,cm.}$ 693 mμ., 160; 618 mμ., 2226; 582 mμ., 1094. $E^{1\%}_{1\,cm.}$ 326 mμ., 661 (corrected), or 378 calculated on oil (Fig. 2).

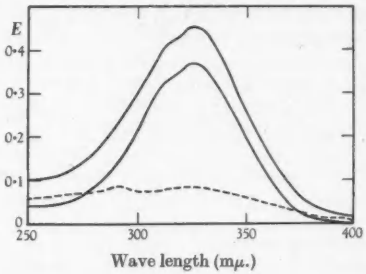

Fig. 2. Absorption curve for unsaponifiable fraction of the tunny-liver oil of Fig. 1, 0·001 %, calculated on weight of oil used; upper curve observed, lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(iv) Unsaponifiable matter (0·2655 g.) was partitioned between 83 % (v/v) aqueous ethanol and light petroleum: (*a*) ethanolic fraction, $E^{1\%}_{1\,cm.}$ 326 mμ., 599 (gross), 434 (corrected), calculated on fraction (0·2086 g.); (*b*) light petroleum fraction (0·057 g.), $E^{1\%}_{1\,cm.}$ 295 mμ., 47; 350 mμ., 22·2; 370 mμ. 16·5; 390 mμ., 8·5. The *E* values are here expressed on the weight of crude unsaponifiable matter; the absorption curve is uncorrectable. The fraction (*b*) seemed to be rich in 'cyclized' vitamin A but free from the vitamin itself. Appreciable losses of vitamin A occurred in this experiment.

(v) After standing for some weeks the small remaining amount of sample A had deteriorated appreciably: $E^{1\%}_{1\,cm.}$ 328 mμ., 403 (gross), 318 (corrected). A portion, 0·1 g., was saponified and the unsaponifiable fraction extracted with 250 ml. of 50 % (v/v) aqueous ethanol by warming on a water bath with frequent shaking. The extract was filtered through kieselguhr, and the combined extract and washings made up to 500 ml. with absolute ethanol. $E^{1\%}_{1\,cm.}$ 325 mμ., 369 (gross); 315 (corrected). The 50 % ethanol thus dissolves all the vitamin A but only a small part of the material contributing irrelevant absorption.

Sample B

(i) Solution in *cyclo*hexane (0·001 %), $E^{1\%}_{1\,cm.}$ 328 mμ., 484 (gross); 397 corrected on the basis of esterified vitamin A as the predominant form.

(ii) A portion of oil (0·05 g.) in 10 ml. peroxide-free ether was poured on to a column of silver sand (20 × 1·5 cm.). The

solution dispersed the oil at the top quarter of the column and the solvent was removed by means of an upward current of nitrogen. The column was then washed twice with 90 ml. portions of 70% (v/v) ethanol-water. (*a*) The first washing was diluted 1 in 5 with absolute ethanol, after first making up to 100 ml. ($c = 0{\cdot}001\%$ calculated on the weight of oil used initially.) $E^{1\%}_{1\,cm.}$ 326·5 mμ., 49·3 (gross), 34·3 (corrected). Maxima at 370 and 392 mμ. due to anhydro-vitamin A were shown clearly. (*b*) The second washing, made up to 100 ml. was diluted 1 in 5 (absolute ethanol). $E^{1\%}_{1\,cm.}$ 326·5 mμ., 13·8 (gross), 9·8 (corrected). Bands at 370 and 392 mμ., were again shown. The column was then washed with five successive portions of 80% (v/v) ethanol-water. Each was made up to 100 ml. and appropriately diluted for spectrophotometric tests. The following results all refer to concentrations expressed in terms of the initial weight of oil:

	$E^{1\%}_{1\,cm.}$ 326·5 mμ.	
	Gross	Corrected
(*c*)	54·2	49·0
(*d*)	51·3	45·5
(*e*)	34·5	31·5
(*f*)	28·6	25·2
(*g*)	20·5	17·5

The column was then washed with absolute ethanol (90 ml.) and the washings made up to 200 ml. A portion was diluted twentyfold. $E^{1\%}_{1\,cm.}$ 326·5 mμ., 200·8 (gross), 156·8 (corrected). By adding together the E values for all the fractions, the total recovery was $E^{1\%}_{1\,cm.}$ 326·5 mμ., 453 (gross), 370 (corrected). A repetition of the experiment gave a total recovery of $E^{1\%}_{1\,cm.}$ 326·5 mμ., 451 (gross).

(iii) A quantity of oil (0·1323 g.) was saponified. The unsaponifiable fraction was extracted with 250 ml. of 50% (v/v) aqueous ethanol by warming on a water bath with frequent shaking for 0·5 hr. The solution was filtered through kieselguhr and the latter was washed with 100 ml. of 50% ethanol. The combined filtrate and washings were made up to 500 ml. with absolute ethanol. The material retained by the kieselguhr was washed by means of redistilled ether into the flask containing the matter insoluble in 50% ethanol. The solvent was removed and the residue dissolved in *cyclo*hexane:

(*a*) Fraction soluble in 50% ethanol. $E^{1\%}_{1\,cm.}$ 325 mμ., 427 (gross), 392 corrected (Fig. 3).

(*b*) Remainder of unsaponifiable matter, $\lambda_{max.}$ 305 mμ., inflexions at 370 and 392 mμ. indicating the presence of 'cyclized' or anhydro-vitamin A; $E^{1\%}_{1\,cm.}$ 326 mμ., 52 (gross), 15 (corrected).

Sample C

(i) $E^{1\%}_{1\,cm.}$ 328 mμ., 395 (gross) in *cyclo*hexane; 323 (corrected) on the basis of esterified vitamin A.

(ii) A quantity of oil (0·1696 g.) was saponified. The unsaponifiable matter was extracted as before with 50% (v/v) aqueous ethanol. The soluble portion was appropriately diluted with absolute ethanol for spectrophotometric examination: $E^{1\%}_{1\,cm.}$ 325 mμ., 357 (gross), 320 (corrected).

Sample D

Four samples representative of another batch of tunny-liver oils were examined in *cyclo*hexane solution and corrected on the basis of esterified vitamin. The results were very close: $E^{1\%}_{1\,cm.}$ 328 mμ., 402 (gross), 334 (corrected).

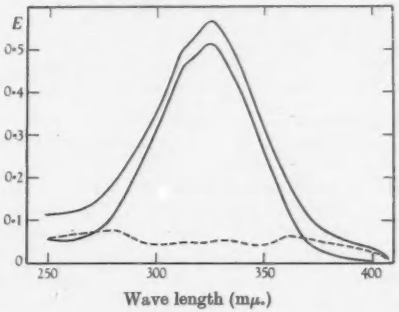

Fig. 3. Absorption spectrum of fraction soluble in 50% ethanol of the unsaponifiable fraction of tunny-liver oil (0·001323% calculated on oil used); upper curve, observed; lower curve, corrected; broken curve, irrelevant absorption. 1 cm. cell.

Discussion of the experiments on tunny-liver oils

The tunny-liver oils examined show exceptionally high values of $E^{1\%}_{1\,cm.}$ 328 mμ., indicating that they contain 40–50% of vitamin A esters. The gross values of the absorption at 328 mμ. determined directly on a *cyclo*hexane solution lead, however, to a considerable over estimate of the vitamin A content because of the presence of material contributing irrelevant absorption. When the spectra are corrected using the gross readings at the fixation points (313, 328 and 338·5 mμ.) appropriate to vitamin A esters in *cyclo*hexane, the vitamin A contribution is seen to be 82–83% of the gross absorption at 328 mμ. The validity of the correction, as already explained, rests upon the assumption that irrelevant absorption is linear over the fixation range.

In order to test the assumption various methods of fractionation have been studied. Partition of the total unsaponifiable material between 83% (v/v) aqueous ethanol and light petroleum led to appreciable losses of vitamin as shown by the results on sample A. In all cases where the unsaponifiable fractions of these oils were freed from the last traces of solvent by means of steam heating in a current of nitrogen, marked losses of vitamin have occurred. It would seem that the free vitamin A as it occurs in the unsaponifiable matter of these very potent oils is more liable to oxidation than the vitamin in the corresponding fractions derived from oils of lower potency.

The method of fractionation suggested by Chevallier, Manuel & Faubert (1941) has only recently become known to us through the courtesy of M. Faubert. It consists of dispersing an oil upon silver sand as an inert support and eluting various fractions by different mixtures of ethanol and water. In trying out the method on sample B, three main fractions were obtained:

(*a*)–(*b*) fraction soluble in 70 % ethanol.
(*c*)–(*g*) fraction soluble in 80 % ethanol.
(*h*) fraction soluble in absolute ethanol.

The possibilities of the method have not yet been fully explored, but for our present purposes the use of sand is not essential.

It has been found that almost all the free vitamin A in the unsaponifiable fraction is readily soluble in 50 % aqueous ethanol. Whereas the corrected *E* value for the oil is 82 % of the gross value it is 92 % of the gross value for the soluble part of the unsaponifiable fraction (see sample B (iii)). Moreover, for samples B and C, the simple correction procedure applied to *cyclo*hexane solutions of the oils, gives the same final result, within very narrow limits, as the fuller fractionation procedure.

The method of fractionation with 50 % aqueous ethanol offers an alternative correction procedure to those workers who lack an accurate photoelectric spectrophotometer. It provides a means for the quantitative recovery of the vitamin A while at the same time eliminating at least half of the irrelevant absorption. It also obviates much of the risk of losing vitamin A in the final stages of preparation of unsaponifiable extracts, since after the removal of ether, the 50 % ethanol can be added at once without trying to get rid of the last traces of ethanol and water.

Conclusions from the experiments on tunny-liver oil

The correction procedure, based on the three point fixation applied to *cyclo*hexane solutions of whole oils, is justified (*a*) by fractionations of the oil and (*b*) particularly by fractionation of the unsaponifiable fraction. If facilities are lacking for the application of the full correction, the best estimate of vitamin A potency is obtained by spectrographic measurements at 325 mμ. on the 50 % ethanol extract of the unsaponifiable fraction.

The results on sample B (ii) show that with both 70 and 80 % ethanol, the amount of vitamin extracted by successive portions of solvent decreases steadily. The oil probably contains a little free vitamin A (easily soluble in 70 % ethanol). A difference is suggested between the esterified vitamin A which is soluble in 80 % ethanol from that requiring absolute ethanol, since the former solvent is progressively less effective in successive elutions despite the fact that more than half the amount of vitamin used is still available. A possible explanation of the difference is that the two fractions differ in respect of the fatty acids with which the vitamin A is esterified. Although the corrected *E* values for the different fractions vary from 70 to 92 % of the gross values, the addition of gross and corrected values shows that 82 % of the absorption is due to the vitamin, a result agreeing with that observed on the whole oil.

Spectrophotometric data for a range of commercial fish-liver oils

The application of the correction procedure to the determination of vitamin A in cod-liver oils has been discussed elsewhere (Morton & Stubbs, 1946). Its bearing on the conversion factor problem has been studied using the halibut-liver oil samples employed in the large-scale co-operative work organized by the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Hume, 1937, 1939; Irwin, 1944). For the fully corrected $E^{1\%}_{1\,cm.}$ value a conversion factor of 1800 is appropriate (Morton & Stubbs, 1947). It is proposed here to summarize experience gained in the use of the method applied to a wide variety of vitamin A-containing materials. Table 2 shows the results obtained for a number of commercial halibut-liver oils examined in *cyclo*hexane solution.

Table 2. *Intensities of absorption shown by commercial halibut-liver oils*

Oil no.	$E^{1\%}_{1\,cm.}$ 328 mμ. Gross	$E^{1\%}_{1\,cm.}$ 328 mμ. Corrected	Vitamin A absorption as percentage of gross absorption
1	28·8	24·2	84·2
2	54·9	47·6	86·8
3	63·4	54·0	85·2
4	39·9	34·3	86·0
5	25·6	21·1	82·4
6	29·1	24·5	84·1
7	14·6	14·0	95·9
8	98·4	95·8	97·4
9	38·0	35·7	94·0
10	26·2	23·4	89·4
11	52·2	46·2	88·6
12	58·2	49·0	84·2
13	37·5	32·1	85·6
14	29·9	28·0	93·6
15	15·0	12·6	84·0
16	25·8	24·3	94·2
			Mean 88·5

For many years the gross $E^{1\%}_{1\,cm.}$ 328 mμ. for halibut-liver oils has been multiplied by a conversion factor of 1600 in order to express the results in international units. When there is no irrelevant absorption the appropriate conversion factor is 1800. Thus only when the corrected *E* value is 88·9 % of the gross

Table 3. *The extent of correction needed in the spectrophotometric measurement of the vitamin A content of fish-liver oils*

Type	No. of samples	$E^{1\%}_{1\,cm.}$ 328 mμ. gross	$\frac{E \text{ corrected}}{E \text{ gross}}$	Average conversion factor for E gross
Very rich oils	12	*c.* 200	0·79–0·89	1500
Rich oils	20	*c.* 100	0·80–0·90	1550
Halibut-liver oils	16	*c.* 40	0·82–0·97	1600
Shark-liver oils	15	*c.* 25	0·77–0·90	1510
Dogfish-liver oils	3	*c.* 6	0·82–0·94	1560
Hake-liver oils	6	*c.* 5	0·85–0·93	1610
Other liver oils	20	*c.* 25	0·70–0·94	1540

value will $E^{1\%}_{1\,cm.}$ 328 mμ. (gross) × 1600 give the true potency. Since Table 2 shows that on the average the corrected E value for halibut-liver oil is 88·5 % of the gross value, 1600 remains the appropriate conversion factor if correction cannot for lack of the right equipment be undertaken. It is quite clear, however, that omission of correction may give rise to serious errors in some cases; the oils most contaminated with artefacts will be over valued and those least contaminated may be very significantly under valued (cf. oils 5, 7 and 8 in Table 2).

Similar results were obtained on a large number of fish-liver oils. In Table 3 many of these oils have been grouped at different potency levels rather than according to species because many commercial oils are obtained from mixed livers.

Throughout Table 3 an average empirical conversion factor between 1500 and 1600 would be justified for use with the uncorrected $E^{1\%}_{1\,cm.}$ 328 mμ. value. The range of variation in the magnitude of the correction again shows, however, that over a period some oils would be over valued and others under valued, but on the average the conversion factor of 1600 has given results surprisingly near the truth.

Spectrophotometric data on commercial molecular distillates (ester concentrates)

As might have been expected, molecular distillates are easily distinguishable from ordinary commercial liver oils. The correction for irrelevant absorption is much smaller and the spectra of some samples examined are within experimental error identical in form with the standard spectrum of crystalline vitamin A acetate dissolved in *cyclo*hexane. It is significant, however, that none of the distillates so far examined require zero or negative correction as would be expected if the observed corrections were all within the experimental error of the procedure (Table 4).

Except for one sample the corrected values are over 92 % of the gross value, and in general the use of a conversion factor of 1700 is justifiable. It is not unlikely that the freshly prepared molecular distillates may have needed little or no correction. Certainly samples 7, 12 and 15 must have been representative of remarkably stable products.

Table 4. *Intensities of absorption of commercial molecular distillates in* cyclo*hexane*

Distillate no.	$E^{1\%}_{1\,cm.}$ 328 mμ. Gross	$E^{1\%}_{1\,cm.}$ 328 mμ. Corrected	Vitamin A absorption as percentage of gross absorption
1	100·7	92·8	92·2
2	102·2	96·6	94·5
3	102·4	95·2	93·0
4	85·8	74·2	86·5
5	85·8	82·6	96·3
6	86·8	84·0	96·8
7	87·6	86·8	99·1
8	87·8	81·9	93·3
9	86·6	80·5	93·0
10	88·2	81·2	92·1
11	86·4	81·2	94·0
12	86·8	85·4	98·4
13	87·2	81·2	93·1
14	86·0	81·2	94·4
15	85·8	84·0	97·9
			Mean 94·3

Spectrophotometric data on ling cod-liver oils

The liver oil of the ling cod (*Ophiodon elongatus*, Girard) and also the commercial oils known as 'red' and 'black' cod-liver oils are interesting in several ways. For marine fish-liver oils they are exceptionally rich in vitamin A_2 as shown by the antimony trichloride colour test. The main selective absorption in the colour test for vitamin A occurs at 617–620 mμ. whereas that characteristic of vitamin A_2 occurs at 693 mμ. In ling cod-liver oils the relative intensities of the two bands are 7–8 to 1, the latter band being the weaker. For many other liver oils from salt-water fish the corresponding ratio is 15–20 to 1. Since the potency of a commercial ling cod-liver oil may be of the order 300,000 i.u./g. the absolute amount of vitamin A_2 present is considerably higher than that normally obtainable from fresh-water fish-liver oils. After saponification and desterolation of the unsaponifiable fraction, a concentrate containing 10 % of vitamin A_2 is obtained.

From the analytical standpoint a study of such oils illustrates the possibility of further extensions of the correction for irrelevant absorption. The ultra-violet absorption curve for a ling cod-liver oil is superficially a normal vitamin A curve (Fig. 4). The criterion that in *cyclo*hexane solution

$$E_{313\ m\mu.} = E_{338{\cdot}5\ m\mu.} = 6/7\ E_{328\ m\mu.}$$

is not, however, satisfied and there must therefore be irrelevant absorption. $E_{338{\cdot}5\ m\mu}$ exceeds $E_{313\ m\mu.}$ in most ling cod-liver oils indicating that the slope

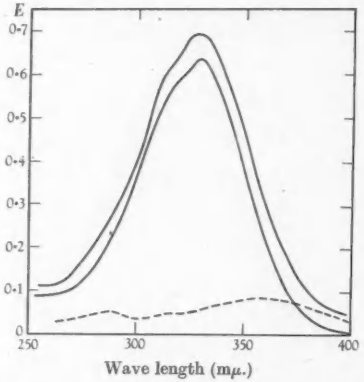

Fig. 4. Absorption spectrum of ling cod-liver oil, 0·0176% solution in *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken curve, subtraction curve showing vitamin A_2 maximum. 1 cm. cell.

of the irrelevant absorption is in the direction opposite to that found for most fish-liver oils. The correction procedure is, however, applicable and a reduced E value for the vitamin A contribution can readily be calculated at 328 mμ. If from the standard curve for vitamin A acetate the whole corrected vitamin A absorption curve is plotted, subtraction from the complete curve (uncorrected) of the true vitamin A contribution will give the irrelevant absorption curve. For most ling cod-liver oils the subtraction curve shows a maximum near 350 mμ. characteristic of the vitamin A_2 present in the oil, and furthermore the ratio $E_{328\ m\mu.}$ (corrected)/$E_{350\ m\mu.}$ (by subtraction) is 7–8 to 1, in very reasonable agreement in most cases with the ratio obtained from the colour test. The agreement is not strictly quantitative because there is some overlapping of the absorptions of vitamins A and A_2 in the colour test. The $E_{350\ m\mu.}$ value is the difference between two comparatively large values and as such is subject to appreciable error. There is no doubt, however, that if the position of $\lambda_{max.}$ for vitamin A_2 were not already known from data on preparations from fresh-water fish-liver oils, the subtraction procedure would have given it fairly correctly. This is of much wider significance than the present problem as it offers a means for arriving at the spectra of contaminants even though the latter may not be chemically separable from the main constituent.

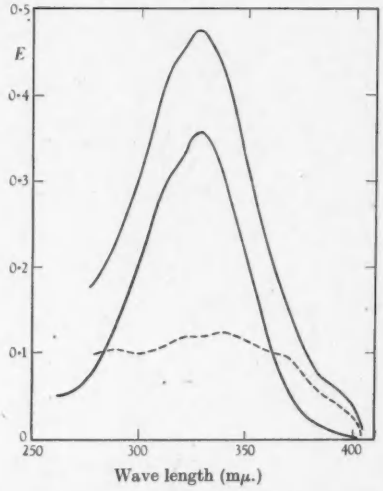

Fig. 5. Absorption spectrum of ling cod-liver oil, 0·015% solution in *cyclo*hexane. In this sample the subtraction curve shows the presence of decomposition products of vitamin A. 1 cm. cell.

Fig. 5 shows the results obtained with a ling cod-liver oil containing 'cyclized' or anhydro-vitamin A as well as vitamins A and A_2. In this case the maximum for vitamin A_2 in the subtraction curve is not so clearly shown owing to the superimposed absorption of the anhydro-product with its maxima at 350, 370 and 392 mμ. The method, however, reveals clearly the spectrum of the principal absorbing contaminant.

Data on whale-liver oils

The assay of whale-liver oils will be dealt with in a separate communication, but the problem may be referred to briefly here.

The spectra of whale-liver products vary rather widely, but in general $\lambda_{max.}$ is well on the short-wave side of 328 mμ. This is the result of very considerable irrelevant absorption due largely in refined oils to kitol with its maximum at 286 mμ. Crude oils are also contaminated with a variety of absorbing materials, especially oxidation products. The accurate assessment of vitamin A content is impossible on the uncorrected absorption, and as the correction

is very variable the use of an empirically based conversion factor leads to nothing better than a first approximation. Correction by our ordinary procedure gives results in surprisingly good agreement with very much more troublesome fractionation procedures. Given a corrected $E^{1\%}_{1\,cm.}$ 328 mμ. value on the oil, or corrected $E^{1\%}_{1\,cm.}$ 326·5 mμ. on the unsaponifiable fraction, the use of a conversion factor of 1800 gives results which are reasonably good.

SUMMARY

1. The absorption spectra of pure vitamin A and vitamin A esters have been determined in *cyclo*hexane, ethanol and 75% (v/v) aqueous ethanol. The results are expressed in terms of the fraction of $E_{max.}$ which corresponds to the absorption at different wave lengths between 250 and 400 mμ.

2. Fixation wave lengths, $\lambda_{max.}$ and two others at which E is 6/7 of $E_{max.}$, have been selected for vitamin A, free or esterified, in specified solvents. By their aid, irrelevant absorption in a liver oil or concentrate is readily detectable.

3. A correction procedure, previously found to be valid for cod-liver oils, has been carefully tested in relation to its validity for high-potency tunny-liver oils. The allowance for irrelevant absorption seems to be given with considerable accuracy.

4. Free vitamin A may be extracted from the total unsaponifiable fraction of fish-liver oils by 50% (v/v) aqueous ethanol. Much of the material giving rise to irrelevant absorption is left behind.

5. Experience gained in testing a wide range of fish-liver oils, including halibut, shark, hake, dogfish and many mixed liver oils shows that irrelevant absorption at 328 mμ. occurs in all, varying from 5 to 20% of the gross absorption. On the average the conversion factor of 1600 was fully justified if irrelevant absorption could not be allowed for, but the use of the factor of 1800 and of corrected E values permits the assay of individual oils with considerably improved precision.

6. Molecular distillates show from 10% to negligible irrelevant absorption. The average for the commercial samples studied is about 5%.

7. The correction procedure applied to the spectra of ling cod-liver oils enables the spectra of vitamin A_2 and anhydro-vitamin A to be obtained at least semi-quantitatively.

8. Crude and refined whale-liver oils are amenable to study by the method, and the results are an improvement upon those of any alternative procedure so far available.

Most of the fish-liver oils examined in the course of this work were obtained through the Ministry of Food.

We are indebted to Dr K. C. D. Hickman and his colleagues (Distillation Products Inc.) for specimens of crystalline vitamin A and vitamin A acetate. One of us (A.L.S.) participated in the work as holder of an I.C.I. Fellowship. The cost of the work has been defrayed from a grant for research obtained through the Scientific Adviser's Division, Ministry of Food, and the earlier studies which led up to the work were supported by the Medical Research Council.

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A High-speed Tissue Homogenizer

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This paper describes a machine designed primarily for preparing uniform dispersions or homogenates of animal tissues in aqueous media for enzyme studies. It has proved invaluable in studies of the arginase and alkaline phosphatase of tissues such as liver, kidney and mammary gland (Folley & Greenbaum, 1946, 1947), particularly in experiments involving estimation of the enzymes in tissues taken from groups of animals killed on the same day where the question of the time required for preparing tissue extracts or homogenates may be crucial.

The technique of Potter & Elvehjem (1936) for preparing homogenates of small fragments of tissue for investigation of the effects of dilution on respiratory enzyme systems was not felt to be suitable for our purpose, which was to obtain estimates of the concentration and total amount of certain water-soluble hydrolytic enzymes in whole organs of the rat. Even though the apparatus of Potter & Elvehjem might well be built on a scale sufficient for 50–60 ml. of fluid, it seemed clear that our work required a relatively robust, yet 'precision' machine, easy to use and capable of dealing rapidly and reproducibly with fair-sized masses of perhaps tougher tissue than one might expect could be easily and routinely handled with the homogenizer of Potter & Elvehjem.

How far our apparatus will be applicable to making tissue dispersions for the study of other tissue enzyme systems and perhaps hormones remains to be seen. Preliminary trials suggest that it may prove useful for homogenizing plant materials, textile fibres, etc.

DESCRIPTION OF HOMOGENIZER

The machine is illustrated in Figs. 1 and 2. It incorporates the principle of rotating knives, a principle which had already been embodied in the Waring Blendor. The homogenization is carried out in the Pyrex glass vessel *A*, consisting of a tube blown at one end into a bulb of 100 ml. capacity, the walls of which are fluted in such a way that the cross section through its greatest diameter is as shown in the lower drawing of Fig. 2. This glass vessel is held firmly and quite centrally inside a cylindrical metal container *B* by the accurately turned fibre collar *C* and the rubber ring *D*, the centre of which is shaped to the bottom of the bulb. Three phosphor-bronze springs *E*, fixed to the inner wall of the container and spaced equidistantly, help to centre the bulb and hold it firmly in position. Vibration during running is minimized by rubber studs inserted into the inner and outer edges of the fibre collar, and projecting slightly so that they press against the outer surface of the glass tube and the inner surface of the metal container respectively.

The tissue to be homogenized is weighed, cut into pieces about the size of a pea with scissors, and placed in the glass vessel together with a suitable volume of the chosen dispersion fluid (saline, Ringer, etc.). The machine works best with a total volume of tissue and fluid of about 50 ml., but in exceptional cases volumes as small as 30 ml. or as high as 60 ml. have been used. Larger volumes cannot be used with vessels of the present dimensions because of the danger of splashing when the machine is running at speed.

The container assembly is put together as shown in the sectional elevation in Fig. 2, and inserted through the collar *F* which it fits accurately. A small dowel on the outside of the container passes up through a slot in the inner face of the collar, the width of which undergoes a slight but gradual increase for about 2 in. in an anti-clockwise direction from the slot, so that on turning the container through about an eighth of a circle with the dowel resting on the top edge of the collar, the flange at the base of the container is brought up tight against the bottom of the collar. The set screw *G* is then tightened to hold the container immobile during the run. The inside of the container is strengthened at the bottom by a metal ring *H* to withstand the pressure of the set screw.

The Universal motor *J* revolves at 12,000–15,000 r.p.m. To its shaft is directly coupled a stainless steel spindle carrying at its lower end four accurately balanced stainless steel knives *K*, two of which are horizontal and two project upwards and downwards respectively at an angle of 45°. Thus a considerable proportion of the interior space of the bulb is swept by the revolving knives, which are of such a length as to pass easily down the neck of the dispersion vessel. The length of the spindle is such that when the apparatus is assembled the two horizontal knives lie below the plane of the greatest diameter of the bulb (*A*—*A* in Fig. 2).

A stainless steel sleeve *L* attached to the spindle overlaps the tapered end of the motor casing *M*, thus preventing oil from the motor bearings creeping down the shaft. At the bottom of the sleeve is fixed a fibre disk *N*, of diameter slightly less than the internal diameter of the neck of the dispersion vessel, to prevent loss of fluid due to splashing. This, however, does not occur if due precautions are taken in starting up.

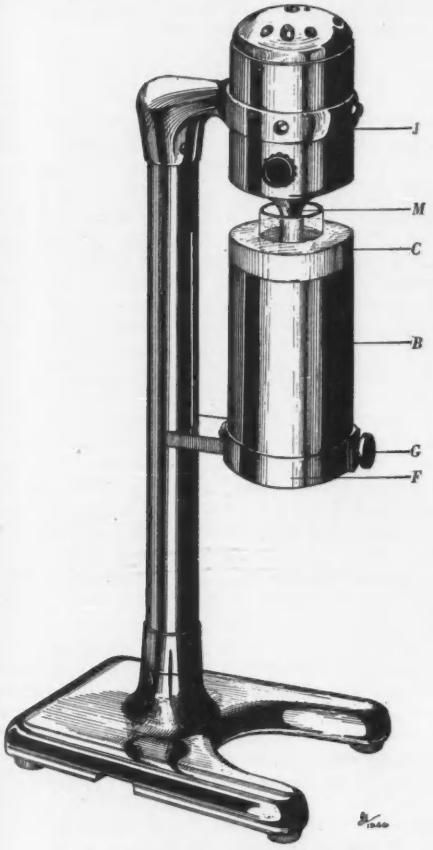

Fig 1. Perspective view of homogenizer.

A suitable sliding resistance is used for starting, and in order to prevent splashing, it is necessary to start slowly, and run at a reduced speed for some seconds until it is obvious from the sound coming from the interior of the dispersion vessel that the lumps of tissue have been largely broken down. Only then is the motor taken up to full speed. If this precaution is not observed the whirling mass of tissue temporarily conglomerated round the knives would tend to force the fluid out of the dispersion vessel. The fluted shape of the bulb is necessary to offer resistance to the rotation of the fluid and to throw it and the tissue into the knives.

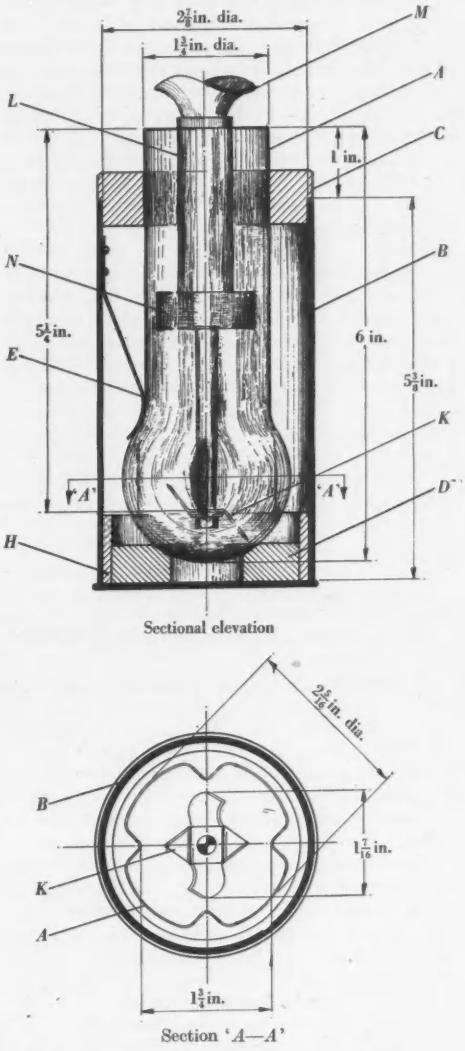

Fig. 2. Sectional scale drawings showing details of dispersion vessel assembly.

Tissues like liver and kidney are uniformly dispersed with the greatest of ease. Stained smears of such homogenates consisted merely of amorphous material quite unrecognizable as belonging to the

organ in question. There were scattered deformed nuclei present but no clear cellular outlines. Occasionally somewhat larger irregular masses could be recognized as containing connective tissue and fragments of blood vessels. The homogenates remain stable for considerable periods and can be pipetted with ordinary pipettes without previous filtration; some sedimentation slowly occurs on standing over some hours, but this heavier material can be redistributed by shaking to give relatively stable homogeneous fluids from which accurate samples can be taken. Lactating mammary tissue also gives good homogenates in 5 min., but these contain small strands of fibrous-connective tissue which should be removed by filtration through glass wool before pipetting. These strands are more prominent in homogenates of involuting mammary gland, which is much more generously interlaced with fibrous-connective stroma, but it is believed that all of the glandular tissue is comminuted. Even with tough tissues like rat's skin, tendon or the cervical region of the uterus, dispersion for 5 min. in this apparatus produces a surprising degree of comminution. These and many other tissues, which the machine can handle with ease, present a formidable proposition when ground with sand in a pestle and mortar.

The contents of the dispersion vessel tend to warm up during homogenization, and if necessary can be cooled by packing the space between the dispersion vessel and the container with ice. Alternatively, it is easy to maintain the tissue at body temperature from the time of removal from the animal until the start of the enzyme determination.

SUMMARY

A machine for the easy preparation of uniform tissue homogenates for enzyme studies is described.

Our best thanks are due to Mr K. C. Richardson for histological examination of tissue homogenates. We are also indebted to Mr K. W. Gladwell of Messrs Nelco Ltd., Shalford, Guildford, for invaluable help with the production of the machine, and to Mr C. Machin for the drawings.

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Component Acids of the Lipids of Human Tubercle Bacilli. Part 1

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Investigations by Anderson and his associates on the chemistry of the lipids of various acid-fast bacilli demonstrated the presence in the bacterial lipids of certain new and specific saturated branched-chain fatty acids of high molecular weight. The acids gave ether-soluble lead salts, and this property was utilized for their isolation from the original mixtures; the separation of the individual acids was accomplished by fractional distillation of the corresponding methyl esters.

Tuberculostearic acid and phthioic acid, isolated by the above procedure from the acetone-soluble fat of the human type tubercle bacillus, strain H-37, appeared to be characteristic components of the lipids of human tubercle bacilli, although the composition of the lipids was found to vary from strain to strain (cf. Crowder, Stodola, Pangborn & Anderson, 1936). More recently studies of cell residues from the preparation of the purified tuberculin protein PPD, derived from an unidentified strain of the human tubercle bacillus, indicated the presence of various branched-chain fatty acids which had not been encountered in previous investigations (Peck & Anderson, 1941; Edens, Creighton & Anderson, 1944; Ginger & Anderson, 1944). According to the last-mentioned paper a dextrorotatory ester fraction, which resembled methyl phthioate (cf. Spielman & Anderson, 1935–6), gave on extended fractionation four fractions varying in composition and in the magnitude of optical rotation.

Investigations in this laboratory (Polgar & Robinson, 1945) led to the synthesis of a number of methyl-substituted long-chain acids, of which 3:13:19-trimethyltricosanoic acid exhibited properties resembling those of phthioic acid. Biological studies by Dr C. E. Coulthard, of the Research Department of Boots Pure Drug Co. Ltd., and Dr J. Ungar, of Glaxo Laboratories Ltd., indicated that the synthetic acid produces in experimental animals lesions similar to the tubercle, the characteristic

lesion of tuberculosis; the activity of the acid in this respect seemed about equal to that reported for phthioic acid.

PRESENT INVESTIGATION

Characterization of fatty acids through semicarbazones of their acetol esters

In view of the above position, further studies seemed desirable. On searching for derivatives, which would facilitate the characterization and purification of such acids, the acetol esters, $R.CO_2CH_2.CO.CH_3$, were found to provide a suitable starting point. Semicarbazones of such esters have been described (Locquin, 1904). In the present investigation, acetol esters of various acids were obtained in almost quantitative yields by refluxing an alkali salt of the acid in ethanolic solution with an excess of chloroacetone. On treatment with semicarbazide, the acetol esters of palmitic and stearic acids gave *semicarbazones* (m.p. 111·5 and 112·5–113·5°, respectively), which were very sparingly soluble in cold ethanol, whereas the *semicarbazones* of acetol 13:16-dimethyltricosanoate and acetol 13:17:21-trimethyldocosanoate (m.p. 67–68° and 70–71°, respectively), obtained in the same way, were comparatively soluble in this solvent, and the semicarbazone of acetol 3:13:19-trimethyltricosanoate was an easily soluble oil. A less soluble and higher melting derivative of the last-mentioned ester could be obtained by treatment with 2:4-dinitrophenylsemicarbazide (McVeigh & Rose, 1945); the resulting *dinitrophenylsemicarbazone* (m.p. 137–138°) was a pale yellow substance which also seemed well suited for application to chromatographic analysis.

The acids could be easily recovered from the foregoing derivatives by alkaline or acid hydrolysis. The acids obtained on hydrolysis of the dinitrophenylsemicarbazones were contaminated with nitro compounds derived from the reagent, but the latter could be readily removed by treating the products with tin and hydrochloric acid, the amines formed by the reduction being easily soluble in the aqueous medium.

Fractionation of fatty acids from human tubercle bacilli

These findings suggested that it might be possible to apply the fractional crystallization of such derivatives to the mixed acids of the tuberculolipids as a step towards isolating the individual acids. The lipids of the residues of human tubercle bacilli were extracted by known procedures and hydrolyzed with ethanolic potassium hydroxide. The acidified product, after removing incompletely hydrolyzed waxy substances by precipitation from an ethereal solution with methanol, was subjected to the scheme of separation described below.

As a preliminary to the application of the fractional crystallization processes, the whole mixture of fatty acids was separated, by fractional distillation of the corresponding methyl esters, into three fractions; each fraction was then converted in the manner already described into acetol esters and thence into semicarbazones. Crystallization of the latter from ethanol gave a sharp separation from very sparingly soluble products which were mainly derived from normal-chain acids. The remaining mixtures were further separated by fractional crystallization and the products so obtained eventually subjected to alkaline hydrolysis to regenerate the corresponding acids.

The ethanolic mother liquors remaining from the above crystallizations contained easily soluble substances of low melting points. After regeneration of the acids from the isolated products, the resulting mixture of acids was oxidized with hydrogen peroxide in formic acid (cf. Swern, Billen, Findley & Scanlan, 1945), the purpose of this operation being to convert unsaturated substances into hydroxylated derivatives. The free hydroxy acids were then isolated by treatment with light petroleum in which they were very sparingly soluble. The remaining petroleum solution contained saturated branched-chain acids which were subjected to further separations by conversion into acetol esters, and then into 2:4-dinitrophenylsemicarbazones. The latter were crystallized from various solvents, and eventually the corresponding acids recovered by hydrolysis of the products. On applying the above scheme to the three methyl-ester fractions obtained from the crude mixture of fatty acids, the lowest ester fraction (b.p. 120–130°/0·5 mm.) appeared to consist mainly of methyl palmitate, and has not been investigated further.

The intermediate fraction (b.p. about 140–160°/0·7 mm.) yielded, on conversion of the acids into acetol esters and thence into semicarbazones, among other products a *semicarbazone* melting at 72–73° which seemed to be derived from a C_{19} acid, in the following referred to as acid A. The regenerated and purified acid was a low-melting solid, m.p. 23–24°; for comparison with tuberculostearic acid it has been further characterized as its amide, m.p. 78–79°, and by the preparation of the tribromoanilide, m.p. 97·5–98°. The melting points of these derivatives correspond within a few degrees to those recorded for the amide and tribromoanilide of tuberculostearic acid (76–77 and 93–94°, respectively; cf. Spielman, 1934), but there is a discrepancy of about 12° between the melting point of the free acids (23–24° as against 10–11°). It may be recalled that Spielman, ascribing to tuberculostearic acid the structure of 10-methylstearic acid, found a similar divergency between the melting point of tuberculostearic acid (10–11°) and that of the

synthetic acid (20–21°), whereas the amides and tribromoanilides had identical melting points. Spielman considered the possibility that tuberculostearic acid may contain, in addition to 10-methylstearic acid, another acid of similar structure, such as 9-methylstearic acid, which could have escaped detection. However, he preferred the explanation that the difference in the melting points of the acids was due to the presence of an asymmetric carbon atom.

In the present experiments, the substances remaining in the mother liquors of the above semicarbazone derived from acid A were hydrolyzed to recover the corresponding acids, and the latter oxidized with hydrogen peroxide in formic acid; after removing hydroxy acids, apparently mainly consisting of dihydroxystearic acid (formed by oxidation of oleic acid), an oil remained which seemed to contain a saturated branched-chain acid different from acid A. It will be necessary to investigate this substance more closely, in order to clarify the position.

The present work was mainly concentrated upon the higher methyl-ester fraction (b.p. about 170–215°/0·7 mm.). This yielded, by application of the same processes, three saturated branched-chain acids, in the following referred to as B 1, B 2 and B 3; besides, a dihydroxy acid, B 4, which was obtained by the use of the hydrogen peroxide-formic acid procedure. Acid B 1 was isolated as its *acetol-ester semicarbazone*, m.p 44–45°. The regenerated acid, which had m.p. 25° and $[\alpha]_D + 4{\cdot}8°$, was further characterized by a series of derivatives, namely, as its *amide*, m.p. 46°, *tribromoanilide*, m.p. 64–65°, and p-*bromophenacyl* ester, m.p. 33–34°. The analytical values indicate $C_{30}H_{60}O_2$ or its near homologues as possible formulae for the parent acid. It may be mentioned in this connexion that a C_{30} acid, designated mycocerosic acid, has been isolated by Anderson and his associates from the waxes of human tubercle bacilli (cf. Ginger & Anderson, 1945), but this acid was laevorotatory, and its *p*-bromophenacyl ester had m.p. 47–48°.

Acid B 2 was obtained as its *acetol-ester semicarbazone*, m.p. 81–82°, from the mother liquors of the semicarbazone derived from acid B 1. The *acid* had m.p. 22° and was also optically active, $[\alpha]_D + 11{\cdot}7°$. It formed an *amide*, m.p. 44°, and a *tribromoanilide*, m.p. 47°. From the analytical figures for these derivatives, the acid may have the composition $C_{27}H_{54}O_2$ or $C_{28}H_{56}O_2$. The acetol-ester semicarbazone derived from acid B 3 was liquid at room temperature and very easily soluble in organic solvents; it remained in the mother liquors which resulted from the fractional crystallizations of the semicarbazones derived from the acids B 1 and B 2. The recovered acid, after removing an unsaturated component (B 4, discussed below) by the hydrogen peroxide-formic acid process, and admixed low-boiling acids by fractionation of the methyl esters, was converted into its acetol ester, and thence into the 2:4-*dinitrophenylsemicarbazone*. The latter had m.p. 153–154·5°, and the analytical figures agreed with those required for phthioic acid, $C_{26}H_{52}O_2$, as the parent acid. The regenerated acid solidified at 0° but melted at room temperature; its specific rotation was about +11°. The amide showed very poor crystallizing properties, and the tribromoanilide was obtained as a low-melting solid which liquefied at room temperature. The acid obviously requires further purification. Kuhn-Roth determination of C-methyl gave 3·4 mol. of acetic acid from acids B 1 and B 3, and 2·9 mol. from acid B 2, as compared with 2·1 mol. of acetic acid in case of acid A. Assuming that the last is a monomethylstearic acid, comparison of the above values would indicate the presence of possibly four terminal methyl groups in case of the acids B 1 and B 3, and three such groups in acid B 2.

Preliminary studies of monolayers of the acids B 1, B 2 and B 3 indicate that only acid B 3 exhibits properties like those characteristic for phthioic acid (cf. Stenhagen & Ställberg, 1941), collapsing on compression at about 40 A.^{2}; acids B 1 and B 2 appear to give unstable films continually collapsing on compression.

It remains to describe briefly the *dihydroxy acid* B 4 which has been mentioned above in connexion with the isolation of acid B 3. It had m.p. 69–70°, and the analytical data were in agreement with a composition $C_{26}H_{52}O_4$ (or near homologues). Oxidation with chromic acid by the Kuhn-Roth method gave 1·6 mol. acetic acid, indicating the presence of two terminal methyl groups.

The results of this preliminary study indicate that a certain separation of the mixture of acids can be achieved by employing the scheme described. However, it will be necessary, in larger-scale experiments, to supplement the above processes by efficient ester fractionations.

For comparison with the above results, the same scheme of separation has been recently applied by the present author to a mixture of acids from the acetone-soluble fat of *Mycobacterium phlei*, a quantity of which was kindly provided by Imperial Chemical Industries Ltd., Dyestuffs Division. The results of these studies will be published in a later communication, but they may be anticipated by stating that the saturated branched-chain acids of higher molecular weight produced by this non-pathogenic member of the acid-fast group appear to be entirely different from those of the human tubercle bacillus.

EXPERIMENTAL

Analyses, including C-methyl determinations, are by Drs Weiler and Strauss, Oxford.

Acetol esters. The following preparation of *acetol palmitate* is typical: A solution of palmitic acid (0·5 g.) in ethanol

(10 ml.) was neutralized with ethanolic KOH, using phenolphthalein as indicator; chloroacetone (0·5 g.) was then added and the mixture refluxed for 2 hr. Next day, water was added and the product collected by ether. The ethereal extract was washed thoroughly with water, and the residue remaining after evaporation of the ether solution heated at 100° under reduced pressure in order to remove completely any unreacted chloroacetone. The resulting ester (yield, practically quantitative) crystallized from ethanol in the form of needles, m.p. 50°. (Found: C, 73·2; H, 11·6. $C_{19}H_{36}O_3$ requires C, 73·1; H, 11·5%.) On treatment with semicarbazide acetate in ethanolic solution it gave a *semicarbazone*, m.p. 111·5° (from ethanol). (Found: C, 65·3, 65·5; H, 10·2, 10·6. $C_{20}H_{39}O_3N_3$ requires C, 65·0; H, 10·6%.)

The following *semicarbazones* were similarly prepared: *acetol stearate semicarbazone*, m.p. 112·5–113·5° (Found: C, 66·7; H, 10·8. $C_{22}H_{43}O_3N_3$ requires C, 66·5; H, 10·8%); *acetol* 13:16-*dimethyltricosanoate semicarbazone*, m.p. 67–68° (Found: C, 70·0; H, 11·3. $C_{29}H_{57}O_3N_3$ requires C, 70·3; H, 11·5%); *acetol* 13:17:21-*trimethyldocosanoate semicarbazone*, m.p. 70–71° (Found: C, 70·25; H, 11·3. $C_{29}H_{57}O_3N_3$ requires C, 70·3; H, 11·5%).

Acetol 3:13:19-trimethyltricosanoate, on treatment with semicarbazide acetate, gave an oil. Its 2:4-*dinitrophenylsemicarbazone*, obtained by short refluxing of an ethanolic solution of the ester with the calculated amount of 2:4-dinitrophenylsemicarbazide, was a pale yellow powder, m.p. 137–138°, after recrystallization successively from ethanol and benzene-ethanol. (Found: C, 64·2; H, 8·75. $C_{36}H_{61}O_7N_5$ requires C, 64·0; H, 9·0%.)

Extraction of the lipids of tubercle bacilli. The bacterial cells used for this investigation were residues from the manufacture of tuberculin (PPD), kindly provided by the Veterinary Laboratory, Weybridge, of the Ministry of Agriculture and Fisheries. They were derived from three human strains of *Myco. tuberculosis*, designated 'mixed P.N., D.T. and C', which were grown on a synthetic medium (glycerol, glucose, asparagine) for 10 weeks, and then killed by the action of steam. The bacterial cells were extracted by successive treatment with ethanol, ethanol-ether (1 : 1), and ether-chloroform (1 : 1) at room temperature. The solutions were decanted, filtered from the bacterial cells, and evaporated under reduced pressure. The residual products were then combined and extracted with ether.

Isolation of the mixed fatty acids. The above ethereal extract was evaporated, and the residue (about 270 g.) hydrolyzed by refluxing for 2·5 hr. with a solution of KOH (40 g.) in water (40 ml.) and ethanol (120 ml.). Under these conditions the fats are hydrolyzed, but the waxes incompletely or only to a slight extent. After addition of water and acidification, the product was collected by means of ether, and waxy substances separated by precipitation of the ethereal solution with methanol. The solution remaining after removal of the precipitate was evaporated and the residue (80 g.) converted into methyl esters by refluxing for 3 hr. with 10% (w/v) methanolic H_2SO_4.

Fractionation of the methyl esters. The following fractions were collected: a low-boiling fraction, b.p. 120–130°/0·5 mm. (7·4 g.); a middle fraction, b.p. 140–160°/0·7 mm. (41 g.); and a high-boiling fraction, b.p. 170–215°/0·7 mm. (8·3 g.).

The low-boiling fraction. This was only tentatively tested. A small sample was hydrolyzed and the acid converted into its acetol ester, and thence into the semicarbazone in the manner already described. The semicarbazone was obtained as a white crystalline powder, m.p. 110–110·5° after crystallization from ethanol. (Found: C, 64·5; H, 10·5. $C_{20}H_{39}O_3N_3$ requires C, 65·0; H, 10·6%.) There was no depression on admixture with the semicarbazone of acetol palmitate; however, this is not of great significance, as the melting points of several acetol-ester semicarbazones derived from normal-chain acids lie close together and are not depressed on admixture. Hydrolysis of the above semicarbazone gave an acid, m.p. 61° alone or mixed with palmitic acid.

The middle fraction. The ester (41 g.) was hydrolyzed by refluxing with ethanolic KOH for 3 hr. Water was then added, and the alkaline solution extracted with ether; a small quantity (0·4 g.) of a non-acidic product was thus obtained. Acidification of the alkaline solution gave a mixture of acids which were isolated by ether extraction. After removal of the ether, the product was warmed with ethanol (50 ml.) and the solution kept for 3 hr. It deposited 5 g. of a solid, m.p. 52–53°, probably mainly a mixture of normal-chain acids.

The remaining solution was neutralized with ethanolic KOH; a large excess of chloroacetone (17 g.) was then added and the mixture refluxed for 3 hr. The product, isolated in the manner already described, was dissolved in ethanol (200 ml.) and brought to reaction with semicarbazide acetate (from 16 g. of semicarbazide hydrochloride and 24 g. of sodium acetate in 20 ml. of water). Next day the precipitated mixture of semicarbazones was filtered off; recrystallization from ethanol gave a sharp separation from a very sparingly soluble product (12 g.), m.p. 98–100°, probably mainly derived from normal-chain acids. On addition of water to the mother liquors a semicarbazone, m.p. 68° (sintering at 63°), separated; after two further crystallizations from aqueous ethanol the product was obtained as minute needles (5 g.), m.p. 72–73°. (Found: C, 66·8, 66·7; H, 10·7, 10·8. $C_{23}H_{45}O_3N_3$ requires C, 67·2; H, 10·95%.) The corresponding acid, regenerated from the semicarbazone by hydrolysis with ethanolic KOH, was a mobile oil. To remove admixed unsaturated substances, the product was heated with formic acid (98%; 10 ml.) and hydrogen peroxide (30%; 2·5 g.) at 60° (bath temperature) with stirring for 1 hr. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH, in order to hydrolyze the formoxy compounds which were formed in the foregoing process. On keeping the free acid with light petroleum (b.p. 40–60°) a small quantity of a substance, m.p. 78°, was deposited. The remaining solution was then evaporated and the residual product esterified by means of methanolic H_2SO_4. The methyl ester distilled as a colourless liquid (2·5 g.), b.p. 157–160°/1 mm., $n_D^{12.5°}$ 1·4480. Hydrolysis of the ester gave the corresponding acid (A), m.p. 23–24°, C-methyl, 10·5%. It gave an amide, m.p. 78–79° (from methanol). (Found: C, 76·5; H, 12·8. Calc. for $C_{19}H_{39}ON$: C, 76·8; H, 13·1%.) The tribromoanilide crystallized from ethanol in needles, m.p. 97·5–98°. (Found: C, 48·8; H, 6·3. Calc. for $C_{25}H_{40}ONBr_3$: C, 49·2; H, 6·6%.)

On concentrating the mother liquors of the semicarbazones described in the preceding paragraph an oil separated. Water was added, and the product isolated with ether. It was hydrolyzed by means of ethanolic KOH, and the acidified product extracted with ether. The product remaining after evaporation of the ethereal extract was then oxidized by hydrogen peroxide (30%; 7 g.) in formic acid (98%; 25 ml.) at 50° (bath temperature) for 1 hr., while

stirring the mixture. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH for 2 hr. After isolation of the acidified product (ether extraction), it was treated with light petroleum (b.p. 40–60°). The substance (5 g.), which separated from the petroleum solution, had m.p. 78°, raised to 93·5° after two crystallizations from 95% ethanol. (Found: C, 68·3; H, 11·05. Calc. for $C_{18}H_{36}O_4$: C, 68·4; H, 11·4%.) From its melting point and analytical values this substance appears to be 9:10-dihydroxystearic acid (m.p. 95°), arising from the oxidation of oleic acid.

The remaining petroleum solution seemed to contain a saturated branched-chain acid; this will be investigated more closely when opportunity permits.

The high-boiling fraction. The ester (8·3 g.) was hydrolyzed with ethanolic KOH. On attempting to remove non-acidic products, by ether extraction of the alkaline solution, emulsions resulted; therefore, the solution was acidified and the product collected with ether. The ethereal solution was then evaporated and the residue kept with ethanol (20 ml.); a crystalline product (0·5 g.) of m.p. 82–83° separated, probably mainly normal-chain acid. (Found: C, 78·0; H, 12·7. Calc. for $C_{24}H_{48}O_2$: C, 78·3; H, 13·0%.) The remaining ethanolic solution was neutralized with sodium ethoxide (0·5 g. of sodium in 10 ml. of ethanol); an excess of calcium chloride in ethanolic solution was then added and after keeping the mixture for several hours, the precipitated calcium salt filtered off. This was decomposed with HCl, and the liberated acid isolated with ether (weight of substance, 4·8 g.). The ethanolic mother liquors of the calcium salt were evaporated and the residue, after treatment with HCl, isolated by ether extraction. The ethereal solution, after washing it first with aqueous KOH and then thoroughly with water, yielded on evaporation 0·8 g. of non-acidic material. The combined alkaline and aqueous washings were acidified and extracted with ether; evaporation of the ether left 1·7 g. of acidic substance which was combined with the material obtained from the precipitated calcium salt.

The above acid (6·5 g.) was neutralized with a solution of sodium ethoxide in ethanol (from 0·5 g. of sodium in 10 ml. of ethanol), and the product brought to reaction with an excess of chloroacetone (7 g.) by refluxing the mixture for 5 hr. The resulting acetol ester was then treated in ethanolic solution with semicarbazide acetate in the usual manner. On crystallization of the crude semicarbazone from ethanol a very sparingly soluble substance (0·22 g.) having m.p. 103–104·5°, probably derived from normal-chain acids, separated first. From the mother liquors two additional *semicarbazones* were obtained by fractional crystallization; one of these (B 1, 1 g.) had m.p. 44–45°. (Found: C, 72·4, 72·1; H, 11·6, 11·7. $C_{24}H_{47}O_3N_3$ requires C, 72·2; H, 11·9%), and the other (B 2, 0·45 g.), which separated on concentration of the combined ethanolic mother liquors of the foregoing semicarbazone, melted at 81–82°. (Found: C, 71·55; H, 11·4. $C_{32}H_{63}O_3N_3$ requires C, 71·5; H, 11·7%.) Further concentration of the mother liquors of this product gave an oil which was isolated by the addition of water, followed by ether extraction. It was then hydrolyzed with ethanolic KOH and the recovered acid (about 4·5 g.) subjected to a repetition of the above processes (conversion into acetol esters, and thence into semicarbazones). A small quantity (0·3 g.) of solid semicarbazones was obtained which on crystallization from ethanol yielded further amounts of the semicarbazones B 1 and B 2; the main product was again an oil. The latter was subjected to the following processes.

The recovered acid was treated with hydrogen peroxide (30%; 2 g.) in formic acid (98%; 12 ml.) at 50° for 1·5 hr. in the manner previously described. After hydrolyzing the resulting formoxy compounds by means of ethanolic KOH, the free acid (isolated by ether extraction) was refluxed with light petroleum (b.p. 40–60°) for a few minutes, and the mixture allowed to stand overnight. It deposited a *substance* (acid B 4, about 0·1 g.), m.p. 69–70° (after recrystallization from aqueous ethanol). (Found: C, 72·7; H, 11·9. $C_{26}H_{52}O_4$ requires C, 72·9; H, 12·15%); C-methyl, 5·5%.

The petroleum solution was then evaporated, and the residual product converted into its methyl ester by refluxing with 10% (w/v) methanolic H_2SO_4 for 3 hr. On fractionation of the ester a fore run (0·85 g.) distilled below 165°/0·7 mm.; the remainder distilled mainly at about 190–210°/0·7 mm. as a viscous oil (2·05 g.), $n_D^{17.5°}$ 1·4620, $[\alpha]_D$ +8·2° (in ether; $c = 11$). The product was hydrolyzed with ethanolic KOH, and the resulting acid converted by the usual procedure into its acetol ester. A solution of this ester in ethanol (40 ml.) was refluxed with 2:4-dinitrophenylsemicarbazide (1·4 g.) for about 10 min., and the solution then filtered hot. Next day the precipitated product was collected and crystallized successively from ethanol and benzene-ethanol. A *dinitrophenylsemicarbazone*, m.p. 153–154·5° was thus obtained as the main product (1 g.) which on hydrolysis, as will be described below, gave acid B3. (Found: C, 63·7; H, 8·6; N, 10·4. $C_{36}H_{61}O_7N_5$ requires C, 64·0; H, 9·0; N, 10·4%.)

Acid B 1. This was obtained from the above semicarbazone, m.p. 44–45°, by hydrolysis with ethanolic KOH. The *acid* distilled at 210–220°/0·05 mm. as a viscous oil (0·76 g.) which quickly solidified and had m.p. 25°; $[\alpha]_D$ +4·8° (in ether; $c = 15·2$); C-methyl, 11·7%. The acid was saturated, and gave an *amide*, m.p. 46° (from methanol) (Found: N, 3·1. $C_{30}H_{61}ON$ requires N, 3·1%.) Its *tribromoanilide*, after three crystallizations from ethanol, melted at 64–65° (sintering at about 60°). (Found: C, 57·0; H, 7·9. $C_{36}H_{62}ONBr_3$ requires C, 56·6; H, 8·1%.) The p-*bromophenacyl* ester had m.p. 33–34° (sintering at 30°) after repeated crystallizations from ethanol. (Found: C, 69·9; H, 9·6. $C_{38}H_{65}O_3Br$ requires C, 70·3; H, 10·0%.)

Acid B 2. Hydrolysis of the semicarbazone previously described, m.p. 81–82°, in the usual manner afforded the crude *acid* which distilled from a bath at 210–220°/0·05 mm. as a viscous oil (0·32 g.). It quickly solidified and had m.p. 22°, $[\alpha]_D$ +11·7° (in ether; $c = 6·5$), C-methyl, 10·5%; the acid was saturated. It gave an *amide*, m.p. 44° (from methanol). (Found: N, 3·25. $C_{28}H_{57}ON$ requires N, 3·3%.) The *tribromoanilide* crystallized from ethanol as short needles, m.p. 47°. (Found: C, 55·0, 55·3; H, 7·6, 7·5. $C_{33}H_{56}ONBr_3$ requires C, 54·9; H, 7·8%. $C_{34}H_{58}ONBr_3$ requires C, 55·5; H, 7·9%.)

Acid B 3. The isolation of the acetol-ester dinitrophenylsemicarbazone derived from this acid has been outlined above. Hydrolysis with a mixture of sulphuric acid, acetic acid and water (5 ml. of each), by refluxing the mixture for 3 hr., afforded the crude acid which was contaminated with nitro compounds. After isolating the product by ether extraction, it was heated with tin (1·2 g.) and HCl (10 ml. of conc. acid and 5 ml. of water) on a steam bath for 1 hr. Water was then added, and the acid isolated by ether. It

distilled at 200–210°/0·05 mm. as a pale yellow oil which solidified on cooling with ice, and melted at about 20°; $[\alpha]_D$ +11·1° (in ether; c = 11·1); C-methyl, 12·8%. The amide was obtained as a low-melting solid which was very soluble in organic solvents; the tribromoanilide separated from aqueous methanol as an oil.

SUMMARY

1. A scheme of separating mixtures of the component fatty acids from the lipids of human tubercle bacilli is described. The scheme, which may be found of value in other connexions, depends upon the fractional crystallization of derivatives, obtainable by converting the acids into acetol esters, $R.CO_2CH_2.CO.CH_3$, and treating the latter with an appropriate reagent for ketones, such as semicarbazide or 2:4-dinitrophenylsemicarbazide.

2. The acids isolated by this process include a saturated C_{19}-acid, resembling tuberculostearic acid in respect of the melting points of derivatives; however, the melting points of the free acids are found to differ by about 12°, and the available evidence seems to indicate the presence of an additional acid of similar composition, having a lower melting-point.

3. From a mixture of the acids of higher molecular weight three dextrorotatory products are obtained by the above procedure; of these, two appear to contain four terminal methyl groups, and the third, three such groups.

4. The presence of an unsaturated branched-chain acid of high molecular weight is indicated by the isolation of its oxidation product; for the oxidation hydrogen peroxide in formic acid has been employed.

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The Determination of Glutathione and other Substances Contained in Yeast

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In the course of an investigation of the action of glutathione on the growth of tissue cells *in vitro* (Astrup & Fischer, 1946) we made some determinations of the amount of glutathione present in Danish yeasts. During these determinations some difficulties were encountered which are of importance also for the determination of other substances contained in yeast and which, as far as we could find, have not previously been described.

METHODS

Glutathione was measured colorimetrically with nitroprusside (Fujita & Numata, 1939; Numata, 1940), using, however, 2N-NH_3 instead of a N-solution for developing the colour, and a standard curve made under identical conditions for comparison. Reduction of GSSG to GSH was carried out electrolytically after Dohan & Woodward (1939). Phosphorus was estimated according to Fiske & Subbarow (1925), using a Pulfrich photometer (Teorell, 1931). Glutathione was prepared by the method of Pirie (1930).

RESULTS

When metaphosphoric acid was used for extracting GSH from yeast no definite results could be reached. Thus, Fig. 1 shows a curve *A* the values of which were obtained by grinding different 1 g. lots of baker's yeast with fine sand in a mortar until a paste was formed, then 4 ml. of 5% (w/v) freshly prepared metaphosphoric acid solution were added and

the mixture allowed to stand for varying lengths of time, 5 ml. of water added and the GSH measured in the centrifuged extract. The results are given as mg. GSH/g. of fresh yeast (Fig. 1).

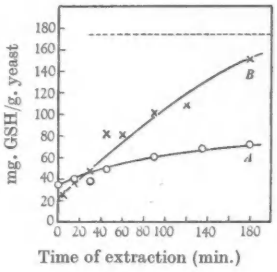

Fig. 1. The extraction of glutathione from yeast by treatment with metaphosphoric and sulphosalicylic acids for various periods. Curve *A*, mg. GSH/g. of fresh yeast obtained by extraction with metaphosphoric acid. Curve *B*, mg. GSH/g. of fresh yeast (another sample) after extraction with sulphosalicylic acid. ----- Total GSH obtained after treatment with ethanol followed by sulphosalicylic acid.

A treatment with sulphosalicylic acid in the same manner yielded a curve *B* of similar appearance, although higher values were obtained. It was observed that a sulphosalicylic acid extract of dried yeast (prepared with ethanol) gave still higher values. The paste from 1 g. of yeast was therefore first treated with 2 ml. ethanol, and then 4 ml. 5 % (w/v) sulphosalicylic acid were added followed by 3 ml. water. The amount of GSH found was now further increased and did not change with time. In this manner the amount marked 'total GSH' in Fig. 1 was obtained. Similar results were obtained when metaphosphoric acid was added after ethanol treatment.

The influence of different concentrations of sulphosalicylic acid on the extraction was investigated (Table 1). The same amount of sulphosalicylic acid/g. of yeast was used, and the concentrations of both yeast and sulphosalicylic acid during the extraction were varied simultaneously. After the treatment water was added until the total volume was 10 ml., the final concentration of sulphosalicylic acid being 2 %. Even when sulphosalicylic acid was present in the highest concentrations during the denaturation period not all GSH was removed, the total amount found after treatment with ethanol and sulphosalicylic acid being 1·86 mg. GSH/g. yeast.

With trichloroacetic acid, however, a treatment with 2 ml. of a 10 % (w/v) solution for 5 min. resulted in total extraction of GSH (1·93 mg. GSH/g. yeast). Addition of ethanol (2 ml.) did not significantly alter this figure (1·85 mg.), while a lower concentration of trichloroacetic acid (4 ml. 5 % solution) during the denaturation was quite insufficient, yielding a value (0·50 mg.) almost as low as that (0·20 mg.) obtained by treatment with 2 ml. 10 % sulphosalicylic acid alone.

Table 1. *Determination of GSH after treatment of* 1 *g. yeast with different concentrations of sulphosalicylic acid*

Sulphosalicylic acid solution added			
Initial concentration (%)	Volume used (ml.)	Water added after extraction (ml.)	GSH (mg./g. yeast)
2·5	8	1	0·25
5·0	4	5	0·47
10	2	7	1·18
20	1	8	1·52
40	0·5	8·5	1·55
100	0·2 g.	8·8	1·65

From these results it is seen that both the nature and the concentration of the denaturing agents are of importance for the extraction. The quantitative determination of GSH in yeast may be performed, according to these investigations, either by treatment of 1 g. of yeast with sand and 2 ml. 10 % trichloroacetic acid for 5 min., or with sand and 2 ml. ethanol followed by 4 ml. of either sulphosalicylic or metaphosphoric acid in 5 % solution. For comparison three 1 g. samples from the same batch of Danish baker's yeast were treated with trichloroacetic acid and with ethanol and sulphosalicylic acid. Trichloroacetic acid treatment gave 2·15, 2·38 and 2·27 mg. of GSH/g. of yeast; while ethanol and sulphosalicylic acid gave 2·25, 2·35 and 2·38 mg. Three types of brewer's yeast were obtained from the Carlsberg brewery: F, U (bottom yeasts) and O (a top yeast). They were washed several times with water and filtered through a sintered-glass filter (G 4) until the dry substance amounted to 25–26 %, corresponding to the dry substance in the baker's yeast used. Yeasts F and U contained 1·40 mg. GSH/g., but yeast O only 0·86 mg. The GSH content was thus considerably lower than in the baker's yeast investigated, in which the amount of GSH in the different samples investigated by us usually varied between 1·80 and 2·30 mg. GSH/g.

In order to see whether the denaturing agents also acted differently on cells other than yeast cells we measured the GSH content of rat's liver. The liver, removed immediately after killing the rat, was kept frozen in a freezing mixture. Samples (1 g.) were

treated as described for yeast. The following results are typical:

Treatment	mg. GSH/g. liver
Trichloroacetic acid (10%)	1·76, 1·74
Metaphosphoric acid (5%)	1·75, 1·85
Sulphosalicylic acid (5%)	1·74, 1·78
Ethanol and sulphosalicylic acid (5%)	1·80, 1·74

They are concordant and independent of the method, and the differences observed with yeast cells must therefore be due to some peculiarity of these cells. It was of interest to find out whether other constituents of the yeast cell would behave in the same manner as GSH. We compared, therefore, the extraction of GSH with that of total phosphorus. The results are shown in Fig. 2. The mode of release of

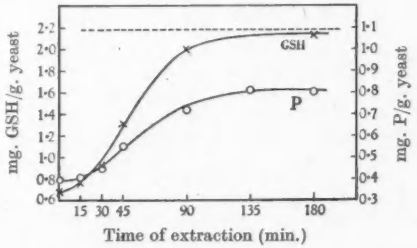

Fig. 2. Comparison of the extraction of GSH and total P from fresh yeast by treatment with 5% sulphosalicylic acid. ----- Total GSH obtained after treatment with ethanol followed by sulphosalicylic acid.

GSH and of phosphorus from the yeast cells is evidently of a similar nature, and the difficulty of obtaining total extraction is most probably due to the cell membrane. When the yeast was treated directly with 5% sulphosalicylic acid without sand, similar curves were obtained, but the values for the first minutes were considerably lower than with previous treatment with sand. The sand may act solely by destroying the cell membrane, so that the cell contents are more easily released. With water alone only very small amounts of GSH, <0·2 mg./g. yeast, were found; even after 3 hr. extraction without sand no phosphorus was found. Treatment with 2 ml. 10% trichloroacetic acid followed by 7 ml. of water yielded after 10 min. without treatment with sand the total amount of GSH and phosphorus. All these results are best explained by the retaining action of the undestroyed cell membrane.

We therefore tried to destroy the cell membrane with some plasmolyzing agents. 5 g. of yeast were treated for 1 hr. with (*a*) 0·25 ml. chloroform, (*b*) 0·25 ml. of Pirie's (1930) mixture (20 ml. conc. sulphuric acid, 100 ml. ethanol and 80 ml. ether), (*c*) 0·25 ml. of benzene, (*d*) 5 g. NaCl, (*e*) 25 ml. of saturated NaCl solution, (*f*) 25 ml. of a 33% (w/v) solution of urea. With benzene and sodium chloride almost no GSH was extracted (about 0·2 mg./g. yeast). Chloroform and urea were considerably better, yielding about 50% of the total GSH. Pirie's mixture, which released almost all GSH, was best.

Repeated freezing of a sample of yeast acted in the same manner. Eight (1 g.) samples of yeast were placed in centrifuge tubes, frozen in a mixture of ether and solid CO_2, thawed and frozen again, the process being repeated six times in all. The yeast became fluid after the first freezing. Then different denaturing agents were added followed by water until the volume totalled 10 ml. The following amounts of GSH were found:

Treatment	mg. GSH/g. yeast
Sulphosalicylic acid (4 ml. 5%)	2·41, 2·36
Trichloroacetic acid (2 ml. 10%)	2·53, 2·36
Ethanol (2 ml.) and sulphosalicylic acid (4 ml. 5%)	2·33, 2·39
Metaphosphoric acid (4 ml. 5%)	(2·91, 2·95)

In this case all the denaturing agents yielded similar results. Metaphosphoric acid was unsuitable as a slight turbidity appeared on addition of the ammonia solution, resulting in too high values for the GSH content.

DISCUSSION

These results suggest that the difficulties met with in determining substances contained in yeast cells may be caused by the resistance of the cell membrane to the action of the various denaturing agents. However, the large differences observed between the action of agents commonly used for the precipitation and denaturation of proteins were unexpected. While trichloroacetic acid yielded the total amount of GSH, sulphosalicylic and metaphosphoric acids did so only after treatment with ethanol. Our use of ethanol originated from the observation that yeast dried with it yields considerably higher amounts of GSH than fresh yeast. Similar observations had already been made with ethanol (Régnier, 1933), and acetone (Schroeder, Collier & Woodward, 1939), in the isolation of GSH from yeast. Treatment of yeast with ethanol in the analytical determination of GSH has been used previously by Weller (1936) and Bolomey (1939), but in connexion with removal of proteins by means of 10% trichloroacetic acid, where according to our experience it is of no use.

The total GSH determined in yeast after electrolytic reduction was often identical with the amount of reduced GSH present, and in no case did the amount rise by more than 10% after reduction. Our

results thus confirm those of Bolomey (1939), and it is our opinion that the large amounts of oxidized GSH found by some authors in yeast may be the result of inaccurate analytical procedures (cf. Fink & Just, 1940).

SUMMARY

1. Different denaturing agents (trichloroacetic acid, sulphosalicylic acid, metaphosphoric acid) release different amounts of GSH and phosphorus from yeast cells.

2. This must be taken into consideration in the estimation of substances contained in yeast cells. Most probably the cell membrane is the cause of the difficulties.

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Preliminary Experiments in the Study of the Respiratory Activity of Micro-organisms Suspended in Thin Films of Fluid Adhering to Solid Surfaces

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It is obvious that the metabolic events taking place in soil occur, under normal field conditions, in the films of water adhering to the soil surfaces. These films vary greatly in extent and thickness, according to soil conditions, e.g. soil structure and moisture content. Evidence secured recently by Lees & Quastel (1946) in their investigations of nitrification in soil show that, even in a soil which is water-saturated but not waterlogged as in their soil perfusion apparatus (Lees & Quastel, 1944), the process of conversion of ammonia into nitrate is brought about by bacteria proliferating at the soil surfaces. It is therefore evident that, for quantitative studies of the biochemical changes taking place in soil, techniques are required which will allow investigations to be made of the metabolism of micro-organisms when these are suspended in films of fluid adhering to solid surfaces, such as those presented by soil.

The well-known Warburg or Barcroft manometric apparatus may be very conveniently adapted for the measurement of the respiration of micro-organisms, when a suspension of these is spread over suitable solid surfaces.

Usually, for estimations of the respiration of micro-organisms, a suspension of the cells in 3 or 4 ml. fluid is placed in the manometer vessel of the Warburg or Barcroft apparatus. The oxygen consumption of the cells, when the apparatus is not shaken, proceeds slowly, the rate being determined by the surface of the fluid in the vessel and the rate of diffusion of oxygen into the fluid. When the apparatus is vigorously shaken new fluid surfaces are constantly exposed to the air in the manometer vessel with the result that the fluid becomes saturated with oxygen. There results an immediate rise in the rate of oxygen consumption by the micro-organisms. The effect of shaking the apparatus is the same as that of spreading the volume of fluid in the vessel over an area so large that the thickness of the film of fluid no longer makes diffusion of oxygen into the fluid a limiting factor. When, therefore, a suspension of the micro-organisms is spread over the surfaces of suitable substances, placed in a vessel attached to a manometer, so that the area of spread is sufficiently great to ensure that diffusion of oxygen into the fluid is not a limiting factor, the resulting rate of oxygen uptake is precisely the same as

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that obtained when the suspension of cells is agitated in the usual way in the Warburg or Barcroft apparatus.

EXPERIMENTAL AND RESULTS

We have demonstrated this fact experimentally by placing in a Warburg manometer vessel various substances offering a large surface, over which a suspension of micro-organisms containing excess nutrient is spread, and by showing that the rate of oxygen uptake is the same, when the apparatus is kept quite steady, as when the suspension is placed in a vessel, in the absence of solid adsorbents, and shaken in the normal way.

As examples of solid adsorbents suitable for the spread of suspensions of micro-organisms for the examination of their respiratory activities in the Warburg respirometer we have used filter paper, pumice, Permutit, Decalso, and air-dried soil. As a test organism we have used a 2% saline suspension of fresh baker's yeast.

Filter paper. A sheet of suitable size Whatman no. 1 filter paper is folded and placed in a Warburg manometer vessel. 1 ml. of a suspension of baker's yeast composed of 0·5 ml. water, 0·2 ml. 10% (w/v) glucose solution and 0·3 ml. of a 2% saline suspension of yeast, is spread drop by drop evenly over the filter paper. 0·3 ml. 20% (w/v) KOH is placed in the inner cylindrical tube of the vessel to absorb CO_2, care being taken that no potash touches the filter paper on which the yeast suspension is spread. In each of two other vessels 1 ml. of the yeast suspension, made up as indicated above, is placed and KOH is put in the inner tubes. No filter paper or other adsorbent is placed in these vessels. All three vessels are attached to Warburg manometers and placed in water baths at 37° and allowed 35 min. to achieve thermal equilibrium. The taps of the vessels are closed after this period. Two of the manometers, one containing the filter paper on which the yeast suspension has been spread and the other containing only the yeast suspension, are not shaken. The third manometer is shaken in the usual way to ensure optimal conditions for aeration of the yeast suspension. The rates of oxygen uptake are recorded in Table 1.

It is necessary, in estimating oxygen uptakes from the manometer readings, to use vessel constants which have been calculated from the expression

$$K_{O_2} = \frac{(V - V_s - V_f)\frac{273}{T} + V_f \alpha O_2}{P_0},$$

where

V = total vol. of vessel,
V_s = vol. of solid adsorbent (mass/density),
V_f = vol. of fluid in vessel,
αO_2 = sol. of oxygen in fluid (μl. gas at N.T.P. dissolved in 1 μl. fluid at partial pressure of gas equal to P),
P_0 = normal pressure = $(760 \times 13{\cdot}6)/D$ (expressed in mm. of manometer fluid where D = density of manometer fluid),
T = absolute temperature.

It is assumed that the solubility of oxygen in the solid adsorbent is nil or so small as to be insignificant.

Table 1. *Respiration of yeast; shaken and unshaken, in the presence and absence of filter paper*

Manometer ...	1 Static 1 ml. yeast suspension in glucose solution spread over filter paper	2 Static 1 ml. yeast suspension in glucose solution	3 Shaken 1 ml. yeast suspension in glucose solution
Time (min.)	Oxygen uptake (μl.)		
15	65	13	64
30	130	26	139
45	193	38	198
60	265	51	268

It will be seen (Table 1) that under the given experimental conditions the velocity of oxygen uptake by the shaken yeast suspension in presence of the excess glucose, is about five times that of the unshaken yeast suspension. The velocity of oxygen uptake of the yeast suspension spread over the filter paper and not shaken is constant and almost identical with that of the shaken yeast suspension.

These results indicate that the film of fluid in which the yeast is suspended, and which has been spread over the filter paper, is sufficiently thin to allow saturation of the fluid with oxygen under the given experimental conditions.

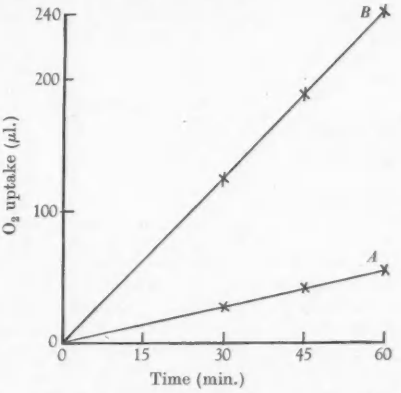

Fig. 1. Rates of oxygen uptake of yeast suspensions (in glucose solution) alone and when spread over pumice. Manometers not shaken. 37°. Air. (*A*) Yeast suspension (in glucose solution) alone. (*B*) Yeast suspension (in glucose solution) spread over pumice.

Pumice. 2 g. of broken pumice (British Drug Houses Ltd. preparation, 10–14 mesh) are placed in a Warburg manometer flask and 1 ml. of the yeast suspension in glucose solution is spread as evenly as possible over the crumbs of the pumice. KOH is placed in the inner tube, and after thermal equilibrium (30–40 min. at 37°) the rate of oxygen uptake is recorded in the usual way, the manometric apparatus, however, being unshaken.

A typical result is given in Fig. 1, which shows also the rate of oxygen uptake of the same volume of yeast suspension not spread over any adsorbent. The rate of oxygen uptake of the yeast suspension spread over the pumice is constant and is identical with that obtained by the suspension when this is shaken in the absence of any solid adsorbent.

These preliminary results would indicate that pumice is a convenient material for the study of the respiration of micro-organisms whose metabolism in films of fluid adhering to a solid surface is the subject of investigation. Air in the manometer vessels may, of course, be replaced by oxygen, if it is thought desirable to test whether the rate of diffusion of oxygen into the pores is a limiting factor.

Permutit and Decalso. The solid adsorbents, Permutit (Permutit Co. Ltd.) (useful as a base exchange material) and Decalso (Permutit Co. Ltd.), may also be used in the Warburg manometric apparatus for the spread of a yeast suspension. Constant rates of oxygen uptake are obtained approximating to that found with a shaken yeast suspension. Results are shown in Table 2.

Table 2. *Respiration of yeast on Decalso and Permutit*

(Warburg manometers static. 37°. Air. Yeast suspension (1 ml. containing 0·5 ml. water, 0·2 ml. 10% glucose solution, 0·3 ml. 2% yeast suspension) placed in a vessel or spread over adsorbents.)

	Manometers not shaken			Manometer shaken
		Decalso	Permutit	
Solid adsorbents...	Nil	(6 g.)	(10 g.)	Nil
O_2 uptake (μl. in 30 min.)	31	148	118	139
O_2 uptake (μl. in 60 min.)	63	292	238	280

Sand. Little or no spread of a yeast suspension takes place over sand, with the result that relatively little increase in the rate of oxygen uptake is obtained in the static manometric apparatus when the cell suspension is distributed over the sand. In one experiment the oxygen uptake in 1 hr. in a static manometer at 37° by 1 ml. yeast suspension (in 2% glucose) spread over Permutit was 240 μl., and by the yeast suspension distributed over 12 g. sand was 99 μl. Thus sand cannot be used as a solid adsorbent for the study of the respiration of yeast, and probably other micro-organisms, suspended in thin films.

Coke. It was thought possible that ground and sieved coke would provide a suitable material for the spread of a suspension of micro-organisms. The specimens of coke we have used have proved to be most unsuitable. When 1 ml. yeast suspension in 2% glucose solution was distributed over 3 g. coke particles, of approximately the same dimensions as the pumice referred to earlier in this paper, the oxygen uptake in 1 hr. at 37° was only 22 μl., less than one tenth of that obtained when the suspension was spread over filter paper. In another experiment, using coke extracted with benzene, acetone and ether, and finally well washed and dried, the oxygen uptake in 1 hr. under the same experimental conditions was 51 μl. Coke, like sand, therefore, may be excluded for the time being from the list of solid adsorbents suitable for the study of respiration of micro-organisms in thin films.

Soil. Air-dried garden soil (4 g.), crumbed and sieved (passing a 2 mm. mesh and retained by a 1 mm. mesh) is placed in each of two Warburg manometer vessels. Yeast suspension (1 ml. composed of 0·5 ml. water, 0·3 ml. 2% yeast suspension in saline and 0·2 ml. 10% glucose solution) is spread drop by drop over the soil crumbs, slowly rotating the manometer vessel to obtain as even a distribution as possible. Glucose solution (1 ml. of a 2%) is spread over the crumbs of the soil in the second manometer flask. KOH is placed in the tubes of the vessels, which are now attached to the manometers and placed in a water bath at 37° to establish thermal equilibrium. After 30–40 min. the taps of the vessels are closed and the rates of oxygen uptake are recorded, the manometers not being shaken. Constant rates of oxygen uptake are obtained and these are recorded in Fig. 2. The rate of oxygen uptake of the 1 ml. yeast suspension in 2% glucose solution spread over 2 g. pumice is also shown in Fig. 2.

There is a fairly considerable oxygen consumption by the soil over which glucose solution alone has been spread, this obviously being due to the micro-organisms present in the soil. The rate of oxygen uptake, secured by the yeast suspension in glucose solution spread over the soil, is much greater than that obtained when the glucose solution alone is added to the soil, and is appreciably greater than that obtained by the yeast suspension in glucose solution spread over the pumice. Examination of the figures recorded in Fig. 2 shows that the difference between the rate of oxygen consumption, in soil, of the yeast suspension in glucose solution, and of that of the glucose solution alone spread over the soil, is almost identical with that obtained by the yeast suspension in glucose solution spread over the pumice. This conclusion would be expected if the yeast suspension spreads as a thin film over the soil crumbs, as has been shown in the case of pumice, and if the oxygen uptake of the yeast in the film of fluid adhering to the soil crumbs is unaffected by the respiration of the soil's own microflora. Other experiments, similar to that whose results are recorded in Fig. 2, have been carried out with yeast suspensions and yield similar results. Since there is no reason at present to believe that in an experiment of short duration the respiration of yeast, in presence of excess of nutrient (glucose) and free

access of oxygen, would be affected by that of the soil's microflora, it follows that soil crumbs may conveniently be used, like those of pumice, for the study of the respiration of micro-organisms in thin films.

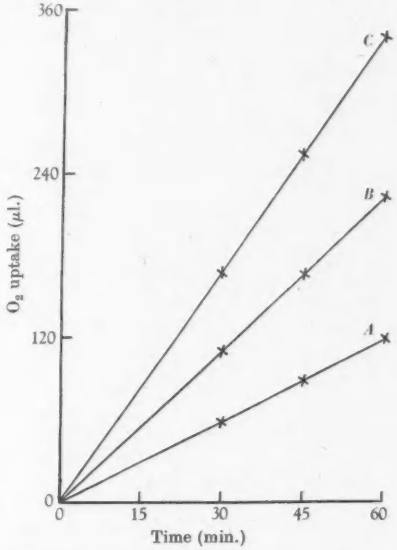

Fig. 2. Rates of oxygen uptake of yeast suspensions (in glucose solution) spread over air-dried soil crumbs and pumice, and of glucose solution spread over soil. Manometers not shaken. 37°. Air. (*A*) 1 ml. 2% glucose solution spread over 4 g. air-dried garden soil. (*B*) 1 ml. yeast suspension (in 2% glucose solution) spread over 2 g. granular pumice. (*C*) 1 ml. yeast suspension (in 2% glucose solution) spread over 4 g. air-dried garden soil.

The fact that the soil has a highly mixed microflora with a variable respiratory activity, depending on the availability of organic substrates, oxygen and water, is of course a complicating factor when studies are being made of the respiration of suspensions of micro-organisms added to soil crumbs. Use, however, may be made of the fact that many micro-organisms have very high respiratory activities, and small quantities of such organisms may secure rates of oxygen uptake far greater than that obtained by the soil alone, on which the suspensions of the organisms are spread.

The adsorbent and the nutrient medium. If an adsorbent alters the constitution of a nutrient medium, by removal of one or more constituents, an effect on the respiration of the micro-organisms under investigation might be expected to take place. So far, in our preliminary experiments, we have not noticed effects of this character. It is clear, however, that our experimental technique should make it possible to explore such effects of adsorbents on the respiratory metabolism of micro-organisms.

Effect of water addition to the solid adsorbents

It is obvious that, as the water content of the adsorbents increases, so will the films of fluid on the adsorbent surfaces increase in thickness. If micro-organisms are suspended in these films, the rate of oxygen uptake of the cells will not be affected by the changed water content until the films assume a thickness where diffusion of oxygen into the fluid becomes a limiting factor. The velocity of oxygen uptake will then fall, the rate of fall being dependent on the rate of waterlogging of the adsorbent. Studies, therefore, of the rates of oxygen uptake of micro-organisms suspended in various volumes of fluid added to a constant quantity of a solid adsorbent will indicate the point at which waterlogging occurs, for at this point there will be a sudden drop in the rate of oxygen consumption. This point, at which waterlogging occurs, is clearly a measure of the water-holding power of the adsorbent; the greater its water-holding power, the greater the amount of fluid which can be added to the adsorbent before waterlogging occurs.

Consequently a greater volume of fluid containing a standard quantity of micro-organisms may be added to an adsorbent of high water-holding power, before a drop in respiration takes place, than can be added to an adsorbent of lesser water-holding power before a similar drop in respiration occurs.

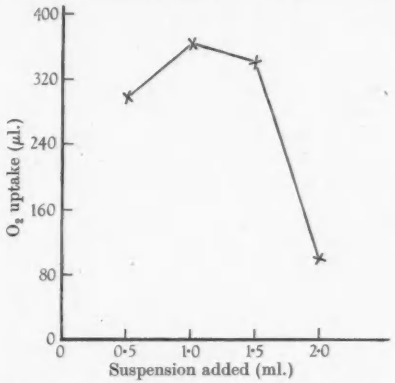

Fig. 3. Variations of oxygen uptakes of yeast suspensions (in presence of glucose) in varying volumes of fluid spread over air-dried soil. Abscissa: Vol. fluid (containing 0·3 ml. 2% yeast suspension and 0·2 ml. 10% glucose) added to 4 g. air-dried allotment soil. Manometers static.

Typical results showing the effects of increased moisture content of soil on the respiration of an added suspension of yeast are shown in Fig. 3.

Respiration is greatest at a moisture content corresponding to the addition of 1 ml. fluid to 4 g. air-dried soil crumbs, i.e. to a water content of 20 % (wet soil). Under the given experimental conditions, greater moisture contents than this lead to the filling up of soil pores with resultant fall in respiration as access of oxygen to the yeast cells becomes more and more restricted. It is noteworthy that under the conditions employed in the experiment, whose results are given in Fig. 3, the highest rate of respiration of the yeast in presence of the soil was 367 μl. O_2 uptake in 1 hr.; that of the soil in presence of glucose solution (1 ml. fluid to 4 g. air-dried soil) was 91 μl. The difference between these two figures is 276 μl. which would be expected to be the oxygen uptake in 1 hr. of the added yeast suspension independent of that of the soil's own microflora. The actual oxygen uptake of the yeast suspension under optimal aeration conditions (shaken Warburg apparatus in absence of soil) was found to be 253 μl. in 1 hr. Our colleague Dr D. M. Webley has investigated the rates of respiration of standard quantities of bacteria of high respiratory activities, suspended in various volumes of fluid, when these are spread over soils (Webley, 1947). Using this technique he has shown how bacterial suspensions may be used for studying problems of aeration into soils. Quastel & Webley (1947) have used this manometric method of assessment of soil aeration for estimating the effects of incorporation with soil of salts of alginic acid and of other organic materials on soil air-water relationships. The addition of sodium alginate to a soil increases its water-holding power; this is shown by the fact that bacterial suspensions spread over soils containing alginate respire at much greater rates at high water contents, than when they are spread over soils containing the same amount of water but with no added alginate. The alginate does not serve as a nutrient source or stimulant to the bacteria, as shown by control experiments; its effect is to afford increased oxygen access to the soil crumbs at the high water contents. The effects of a variety of other substances, e.g. straw, farmyard manure, sawdust, cellulose derivatives, which are all of agricultural importance as organic fertilisers, on the water-holding power of soils may be compared in this way.

SUMMARY

1. If a small volume of a yeast suspension in glucose solution is spread over a suitable solid adsorbent, placed in a Warburg or Barcroft manometer apparatus which is kept unshaken throughout the experiment, its rate of respiration is constant, and is identical with that of the yeast suspension in glucose solution when this is shaken in the normal way in the absence of solid adsorbents.

2. Suitable materials for the spread of suspensions of yeast, and presumably other micro-organisms, so that their respiratory activities in thin films of fluid can be examined, are pumice, Permutit and Decalso. Filter paper also offers an excellent medium for the spread of a suspension of organisms for the study of their respiratory activities.

3. The rate of respiration of a small volume of yeast suspension in glucose solution spread over soil crumbs is constant and greater than that obtained when the suspension is spread over pumice or filter paper. If the rate of respiration due to the soil's own microflora in presence of glucose is subtracted from the rate of respiration of the yeast suspension in presence of glucose spread over the soil, a value is found which is identical with that obtained by the yeast suspension spread over pumice.

It is concluded that soil crumbs, like pumice, form a suitable medium for the spread, as thin films, of small volumes of suspensions of micro-organisms. Soil crumbs may therefore be used for investigations of respiratory activities of micro-organisms suspended in thin films of fluid.

4. Sand and coke are unsuitable materials for the spread of yeast suspensions.

5. The effects of altering the water content of a solid adsorbent on the respiratory activity of micro-organisms spread over the adsorbent are pointed out. A means is indicated of utilizing measures of rates of respiration of organisms suspended in varying volumes of fluid and spread over a standard quantity of solid adsorbent for the study of the water-holding power of the adsorbent, and the aeration conditions within it at various moisture contents. The applicability of this to soil is discussed.

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Further Tests on Human Enamel Protein

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In previous work (Pincus, 1936, 1939) it was shown that human enamel protein differs from the keratins amongst which it had been classed (Rosebury, 1930). It contained 12·1% N, 1·2% S, while cystine was either absent or present in low concentration; tyrosine was also present. Enamel protein resisted digestion both by pepsin and trypsin, and was unaffected by KCN, Na_2S, and sodium thioglycollate. This limited examination has now been extended.

METHODS

Preparation of material. Unerupted human molar teeth were used for experiments throughout; these buried teeth were removed surgically from patients aged from 18 years upwards, and kept at room temperature in water saturated with chloroform. The growth of moulds and bacteria has been avoided in the present experiments by using the protein within a few days after extraction of teeth.

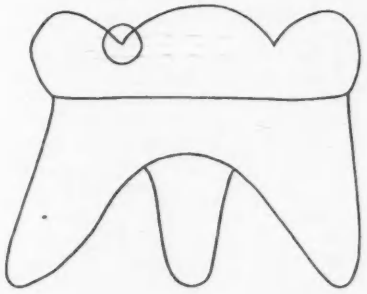

Fig. 1*a*.

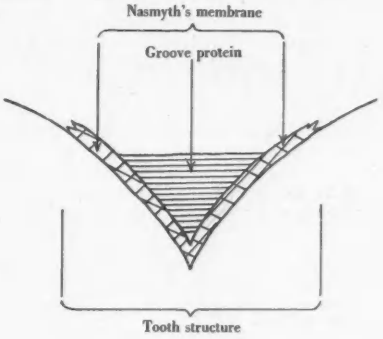

Fig. 1*b*.

Fig. 1 (*a*). Diagram of molar tooth. Position of occlusal groove is marked by a circle. (*b*) Diagrammatic enlargement of circle in (*a*); the positions occupied by Nasmyth's membrane and groove protein in the occlusal groove are indicated.

The teeth were brushed freely with 0·5% Na_2CO_3 solution and with water, the roots separated from the crowns and discarded. Protein was separated from enamel by steeping in 0·5 M-HCl for 24 hr. at room temperature. Dentine was then discarded, and the tiny collections of protein washed in water. Tests have all been carried out on specimens about 3 or 4 mm. diameter at their largest and probably less than 0·1 mm. thick. Prior to 1939, microscopic examination had suggested that the contents of grooves on the occlusal (biting) surface of molar teeth sometimes exceeded the mere surface layer known as Nasmyth's membrane (Nasmyth, 1839). This appearance was thought to be due to debris of section cutting. Table 1 shows results at this stage of the work. However, the existence of two parts in enamel protein has now been recognized, and separate examination of them has been carried out. After the exposure of the teeth to acid (as above), the protein sometimes falls away from the enamel, lying loose. When this occurs, Nasmyth's membrane is seen as a diaphanous structure, while the groove protein is more nearly a tiny solid mass, sometimes of yellowish tinge. Separation was carried out visually under water by the use of a smooth-ended glass pipette 6–8 cm. long, and about 7 mm. diameter. The original name, Nasmyth's membrane, has been retained for the surface layer, i.e. the protein adjacent to the enamel surface; the other part is termed groove protein. Fig. 1 shows the relation of these structures to each other and to the tooth.

RESULTS

Three N estimations shown in Table 1, carried out on mixed Nasmyth's membrane and groove protein, average 11·8%. The low N and the presence of carbohydrate (see Table 2), provide some evidence as to the biochemical nature of these structures; in

considering the estimations, difficulties met in separating tooth proteins from Ca salts must be borne in mind. The N estimations may be compared with those given by Levene (1925) for mucoproteins, where values range from 6·7 % in oviduct (Giacosa, 1882) to 13·3 % in submaxillary glands (Hammarsten, 1888).

Table 1. *Analysis of Nasmyth's membrane mixed with groove protein*

(*a*) Quantitative tests (as %, corrected for ash)

	(Schoeller)	(Weiler)
N	12·1	11·1, 12·1
S	1·2	—

(*b*) Qualitative tests (hydrolysis at 108°, 30 min., pH 8·0)

Xanthoproteic	+
Biuret	– or faint

Table 2 shows that differences of a biochemical nature exist between the two proteins in occlusal grooves. Cupriethylenediamine (CuEn) stains Nasmyth's membrane black, and there is some reason to believe that a Cu protein complex is formed. Some CuEn protein complexes have been examined, and have been found to be bacteriostatic and possibly bactericidal. The groove protein dissolves immediately in CuEn. The purple colour seen on acid hydrolysis of groove protein is absent when Nasmyth's membrane is subjected to acid hydrolysis; the Molisch test is positive with groove protein and negative with Nasmyth's membrane. Cupriammonium solutions dissolve groove protein immediately while Nasmyth's membrane resists for days and is stained black. Since the Molisch test shows the presence of carbohydrate, the possible origin of this carbohydrate is of interest. Glycogen was shown by Glock (1940) to be very abundant in mouth epithelium and in other structures adjacent to the developing molar teeth of foetal and very young rats. Examination for the presence of pentose (in the present work) proved negative. Symington & Rankin (1908) have shown X-ray pictures in which human molar teeth may be seen calcifying downwards from the tip of each cusp, the sides eventually meeting to form a groove. Coalescence of cusps to form a complete occlusal surface occurs normally; and in such a process, developmental material may be 'trapped' in the groove.

While relatively few suitable teeth are available, a rigid selection of material ensures that films of mucin or other adventitious material are kept at a minimum. It is obvious that the tenuous layer on the surface of newly formed enamel, Nasmyth's membrane, is soon removed from exposed surfaces by wear, while in secluded positions such as narrow fissures on the occlusal surface, it may persist. This persistence may also apply to such other contents of fissures as are described above, and may lend to the present work an added value. Such narrow fissures are a very common site of dental caries.

Table 2 shows that exposure to Schweitzer's solution results in histological differences in the structure of groove protein becoming apparent. One part disappears promptly; this is thought to be the remnant 'caught up' as it were during the formation of the tooth, in the groove between the cusps, and

Table 2. *Qualitative tests on Nasmyth's membrane and groove protein separately*

	Nasmyth's membrane	Groove protein
Conc. HCl at 37° for 24 hr.	Dissolves	Dissolves; purple colour
Conc. HCl; boiling water bath	Dissolves >30 min.	Dissolves <2 min.; purple colour
Millon	+	+
Ninhydrin	–	Not tested
Pepsin at 37°	Very slow attack	Very slow attack
Trypsin at 37°	Very slow attack	Very slow attack
Gram – bacillus at 37° (see note *a*)	No attack seen in 14 days	Not tested
Lactobacillus acidophilus at 37°	Not tested	No attack seen in 14 days
Schweitzer's solution at room temperature	Resists	The greater part dissolves; a small part resists (see note *b*)
Cupriethylenediamine at room temperature	Resists for days and is stained black	Dissolves in few seconds
Thioglycollic acid at room temperature	Resists 24 hr.	Dissolves in about 1 min.
Acetic acid	Swells	Not tested
Formol (10%)	Toughened	Toughened
Molisch	–	+
Aniline acetate	–	–
Phloroglucinol	Not tested	Red colour; ppt. No pentose detected spectroscopically

(*a*) This Gram – bacillus was isolated from dental carious lesions (Pincus, 1948), and its proteolytic action on some proteins (e.g. serum, sericin) at 37° has been studied. The rise in free amino groups which is noted during the first week or so of digestion is sometimes followed by a decrease, due to subsequent bacterial degradation. Estimations were thus made over the period where the titre of free amino groups was rising, i.e. where the proteolytic power of the organism could be demonstrated.

(*b*) Microscopical examination showed markings resembling enamel prisms, with a few epithelial cells.

persisting there. This remnant is of mesodermal origin. That part of groove protein, which resists exposure to Schweitzer's solution, is probably a fragment of Nasmyth's membrane which existed in histological continuity with the groove protein, and was inadequately separated from it by the use of a pipette, and the gentle suction used.

In its resistance to chemical and bacterial attack, and to pepsin and trypsin, some resemblance between Nasmyth's membrane and silk fibroin had been pointed out to the writer by the late Sir Patrick Laidlaw prior to 1939. Later, the work of Takamatsu (1933) came to notice, describing the solution of silk in cupriethylenediamine, after which a test of the effect of this substance on enamel proteins seemed to be of interest. Clinical trial of local application of cupriammonium solutions for caries prevention is contemplated, when a suitable technique has been evolved.

SUMMARY

1. Human dental enamel protein has been shown to consist of two proteins, Nasmyth's membrane and groove protein; results of some tests on these are given.

2. Both contain tyrosine, and both resist enzymic attack. Groove protein contains carbohydrate, possibly glucose. In cupriammonium solutions the two structures are sharply differentiated, Nasmyth's membrane resisting solution and staining black, while groove protein readily dissolves.

The writer wishes to thank Professor Margaret Murray for help, Dr Howitt for supplies of cupriethylenediamine and Mr Taylor for the supply of clinical material. To the Westminster Hospital Medical School, which has kindly provided facilities, and the Medical Research Council, which has provided grants, the grateful thanks of the writer are recorded.

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Properties of Glucose Oxidase (Notatin)

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(*Received* 7 *July* 1947)

A glucose oxidase was discovered by Müller (1928, 1936) in *Aspergillus niger* and *Penicillium glaucum*. He found that this enzyme catalyzes the oxidation of glucose to gluconic acid by means of molecular oxygen, and that at a much slower rate it also oxidizes mannose and galactose but not other sugars. Since the activity of this enzyme was found by him not to be affected by cyanide he concluded that it does not depend upon the presence of an active heavy metal.

The enzyme was reinvestigated by Franke & Lorenz (1937) and by Franke & Deffner (1939) who, working with a more purified preparation, demonstrated that during the catalytic oxidation of glucose to gluconic acid, O_2 is reduced to H_2O_2. They found also a certain proportionality between the activity of different preparations and their flavine content and concluded that the enzyme must be a flavoprotein. Although their enzyme preparation was 80 times more active than that of Müller, we now realize that it was not more than 10% pure. Unlike Müller they found that in addition to O_2 other substances such as indophenols may also act as hydrogen acceptors although their efficiency is much lower.

Müller (1941), from a study of the oxidation of various sugars by his enzyme preparations both in O_2 and anaerobically using 2:6-dichlorophenolindophenol, concluded that two enzymes were present, one reacting directly with O_2 and a second which reacts only with certain other H acceptors. Franke (1944) has criticized this conclusion of Müller and also those of Ogura (1939) who claimed to have isolated from *Aspergillus oryzae* a glucose-oxidizing enzyme which could not utilize O_2 as H acceptor. According to Franke (1944) the glucose oxidases from *Penicillium glaucum*, *Aspergillus niger* and *A. oryzae* are virtually identical and consist of a single aerobic oxidase. The findings of Müller (1941) and Ogura (1939) may have resulted from the low purity of their preparations; their Q_{O_2} values being of the

order of 100 at 25° in air, whereas Franke was able to prepare a much purer material ($Q_{O_2} = 8000$ at 30° in O_2). Moreover, the highly purified oxidase ($Q_{O_2} = 130{,}000$) that we have used for the experiments described in this paper can utilize both O_2 and 2:6-dichlorophenolindophenol as H acceptors.

The almost complete purification of glucose oxidase from culture media of *Penicillium notatum* was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, (1942, 1945). They demonstrated that the antibiotic 'notatin', which is thermolabile and only effective in presence of glucose and O_2, is identical with the glucose oxidase of Müller and that its bactericidal action is due to H_2O_2 produced during the oxidation of glucose: $C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2$. The same conclusion was arrived at independently by van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) who purified this enzyme and described it under the name Penicillin B. Their experiments suggested that the prosthetic group of the enzyme was the alloxazine-adenine dinucleotide. Working with the highly purified enzyme we were able to demonstrate that this dinucleotide is indeed the prosthetic group of notatin (Keilin & Hartree, 1946).

Glucose oxidase, notatin and penicillin B therefore designate the same enzyme which catalyzes the aerobic oxidation of glucose to gluconic acid. As a matter of convenience we shall use in our descriptions the short term notatin.

The first part of this paper will deal with the constitution and physical properties of notatin while the second part will consist of a general account of the kinetics of the pure enzyme.

EXPERIMENTAL

A. *Physical properties and constitution of the enzyme*

Notatin* was obtained as a pale yellow powder which dissolved freely in water to give a bright yellow solution with a faint opalescence. On centrifuging, a perfectly clear solution was obtained plus a small purplish residue constituting $< 10\%$ of the preparation.

Absorption spectrum and equivalent weight. A solution of notatin in water containing 10·0 mg./ml. was centrifuged to remove insoluble impurities. It then contained 9·12 mg./ml. This solution was diluted with half its volume of 0·1 M-phosphate buffer pH 6·5 and the absorption curve determined in a 1 cm. layer (Curve I, Fig. 1) using a Beckman photoelectric quartz spectrophotometer. A typical flavine type of absorption spectrum was obtained with maxima at 377 and 455 mμ. van Bruggen *et al.* give the positions of these maxima as 375 and 452 mμ. Addition of glucose to the solution in a closed vessel gives rise to the autoxidizable dihydro or leuco form of the enzyme which is practically colourless (curve II). A more complete reduction to the leuco form was obtained in presence of $Na_2S_2O_4$ (curve III) though the strong absorption by this salt in the ultraviolet region precludes any determination of absorption below 400 mμ.

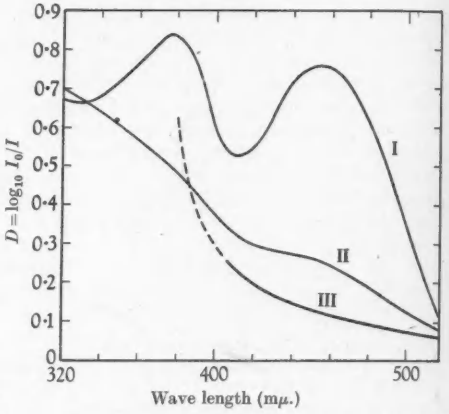

Fig. 1. Absorption spectra of a 0·608% solution of notatin in 0·03 M-phosphate buffer pH 6·5. I, without reducing agent. II, +glucose. III, $+Na_2S_2O_4$.

The absolute value of the height of the absorption band near 450 mμ. is fairly constant for all flavoproteins and the average value of the absorption constant is $\beta = 2{\cdot}4 \times 10^7$ (Straub, 1939). From this figure it is possible to estimate the minimum molecular weight of the enzyme; i.e. the weight of enzyme containing 1 mol. of the alloxazine-adenine dinucleotide. Thus $\beta = (2{\cdot}303\ DM)/cl$, where D = optical density $(0{\cdot}76) = \log_{10}$ incident light/transmitted light, M = molecular weight, c = concentration in g./ml. (0·00608), and l = optical depth in cm. The calculated value of M is 83,000 assuming the enzyme to be completely pure. The correction for colourless impurities will lower this figure while flavoprotein impurities will probably not change it. From the experiments described below it appears that the flavine-free impurities do not exceed 10%, hence the equivalent weight is about 75,000 and the molecular weight a simple multiple of this figure. As the molecular weight of this enzyme determined by Ogston & Cecil (see addendum to this paper) was found to be about 152,000, each molecule of enzyme contains two prosthetic groups.

Coulthard *et al.* determined the absorption spectrum of a similar highly purified preparation and deduced a purity of 90% from the absorption at 450 mμ. Whereas their value of the height of

* The notatin used in these experiments was kindly supplied by Dr W. F. Short (Coulthard *et al.* 1945).

this band agrees very closely with ours, they obtained a considerably higher figure for the second band near 380 mμ.

Electrophoresis. Fig. 2 summarizes the results obtained in the Tiselius electrophoresis apparatus. The material is seen to be almost homogeneous. A small fraction migrates more rapidly than the oxidase and a trace of a more slowly moving fraction can just be detected. Further evidence on the homogeneity of the oxidase was obtained from its behaviour in the ultracentrifuge (see addendum to this paper).

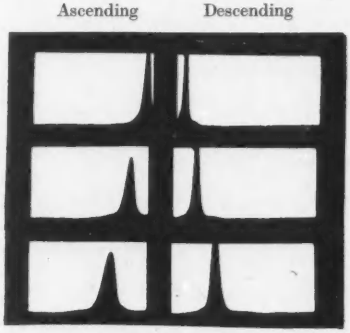

Fig. 2. Migration of glucose oxidase in the Tiselius electrophoresis apparatus. 1·67% solution. 0·05 M-phosphate buffer pH 6·42. Current 19 milliamp. at 250 V. Temp. = 1·5°.

Exposure no.	Time (min.)	Schlieren angle
1	28	65°
2	49	65°
3	80	55°

Nature of the prosthetic group. From the work of van Bruggen *et al.* (1943) it can be concluded that the prosthetic group is probably the alloxazine-adenine dinucleotide (A.A.D.) common to diaphorase, D-amino-acid oxidase and most other flavoproteins. It has not so far been possible to split off the prosthetic group of glucose oxidase and at the same time obtain the protein in a native state. Were it possible to obtain the native protein, then a restoration of catalytic activity on addition of A.A.D. would identify the latter as the prosthetic group. The alternative method, as was suggested by Coulthard *et al.* (1945), is to add the prosthetic group of notatin to the native protein of D-amino-acid oxidase. The prosthetic group of D-amino-acid oxidase is known to be A.A.D. (Warburg & Christian, 1938), hence, as the addition of boiled notatin to the amino-acid oxidase protein restores the specific catalytic activity of the latter (Keilin & Hartree, 1946) the two enzymes must have a common prosthetic group.

The D-amino-acid oxidase protein was separated from its prosthetic group according to the method of Negelein & Brömel (1939) and the enzyme was then reconstructed from 5 mg. free protein (fraction B of Negelein & Brömel) and varying volumes of a standard solution of A.A.D. containing 0·65 mg. riboflavin phosphate/ml. The catalytic activities of the mixtures were estimated manometrically by the rates of oxidation of DL-alanine in pyrophosphate buffer pH 8·3 at 39° in presence of catalase. The relationship between the volume of A.A.D. solution added and the catalytic activity of the system is shown in Fig. 3. The activity of the amino-acid oxidase protein was now tested under the same conditions with the prosthetic group of notatin in place of the A.A.D. solution. For this purpose a solution of notatin in water containing (after centrifuging) 2·77 mg./ml. was heated at 100° for 15 min. and cooled. The addition of 0·02 and 0·04 ml. of this solution to the above oxidizing system brought the amino-acid oxidase activity to the same level as would the additions of 0·00049 and 0·00102 ml. respectively of the standard A.A.D. solution (Fig. 3).

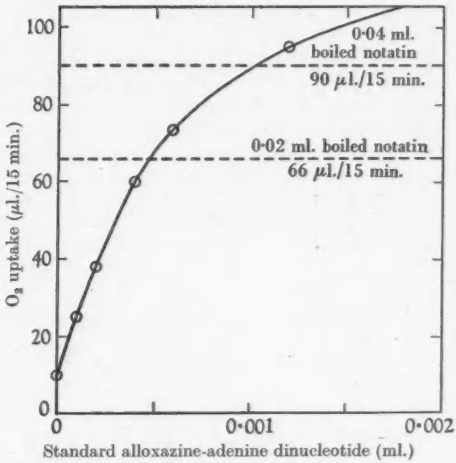

Fig. 3. Rates of O_2 uptake obtained on adding various amounts of alloxazine-adenine dinucleotide or of boiled notatin to the system: 5 mg. D-amino-acid oxidase protein, 12 mg. DL-alanine, 0·1 ml. catalase, 0·07 M-pyrophosphate buffer pH 8·3 to 3·3 ml. Temp. = 39°. Standard dinucleotide solution for calibration (circles) contained 0·65 mg. riboflavin phosphate/ml.

Hence 1 ml. boiled notatin solution containing 2·77 mg. notatin is equivalent to 0·025 ml. of the standard A.A.D. or to 0·0163 mg. riboflavin phosphate. The sample of notatin therefore contains 0·59% riboflavin phosphate. Pure diaphorase contains 0·66% riboflavin phosphate (Straub, 1939), hence if we assume that the riboflavin contents of diaphorase and notatin are the same, and that all the

flavine in the preparation is present in the form of the enzyme, then the notatin is about 90% pure. On the other hand, if the notatin contains flavoprotein impurities this estimate is too high. Thus Ogston & Cecil (addendum to this paper) estimate the purity to be 80% or possibly more, whereas the agreement (above) between their value of the molecular weight and our estimate of the equivalent weight, based on the assumption that there are no flavoprotein impurities, is very close. The purity of the notatin is therefore between 80 and 90%.

Effect of temperature and pH *on the fluorescence of notatin.* Pure notatin does not fluoresce in ultraviolet light although the strong green fluorescence of A.A.D. appears when the enzyme is denatured. In this respect it differs from diaphorase which fluoresces both before and after denaturation. At temperatures below 60° no fluorescence can be detected while at 70° it develops very rapidly. Experiments were carried out at intermediate temperatures in an attempt to reverse the phenomenon by sudden cooling. In no case was there a decrease in the fluorescence produced by the higher temperature.

The effect of pH on the fluorescence is parallel to its effect on enzyme activity (see below). The solution becomes fluorescent at pH >8 or <2, i.e. outside the range of catalytic activity. Here again there was no evidence of reversibility of the appearance of fluorescence or of enzyme activity on rapid neutralization to pH 5. Coulthard *et al.* (1945) showed that the bactericidal properties of notatin rapidly disappear outside the pH range 2–8.

B. *Kinetics of the enzyme reaction*

It was shown by previous workers that oxidation of glucose to gluconic acid catalyzed by glucose oxidase is accompanied by reduction of O_2 to H_2O_2 (p. 221). The formation of H_2O_2 in this primary oxidation reaction has been confirmed by the addition of azide-catalase which rapidly turns from greenish brown to red and changes the pattern of its absorption spectrum (Keilin & Hartree, 1945*a*). It can also be demonstrated by the utilization of this peroxide in a secondary or coupled oxidation of alcohols catalyzed by catalase (Keilin & Hartree, 1945*b*).

Notatin was made up as a 0·1–0·2% aqueous solution, which was stable for 1 week at 5°, and suitable dilutions were prepared as required. Measurements of O_2 uptake were carried out in air in Barcroft differential manometers at 39° (unless otherwise stated). As a general rule the right-hand flasks of the manometers received 0·3 ml. notatin, 0·3 ml. glucose, 0·1 ml. catalase (Keilin & Hartree, 1945*a*) and buffer to 3·3 ml. The left-hand flasks received 3·3 ml. buffer.

Influence of pH *and of buffer concentrations.* The O_2 uptake of the system composed of 15 μg. notatin, 0·3 ml. 10% glucose and 0·1 ml. catalase was determined in various buffers over the pH range 2·3–10·3 (Fig. 4). The buffer concentrations were about 0·2M

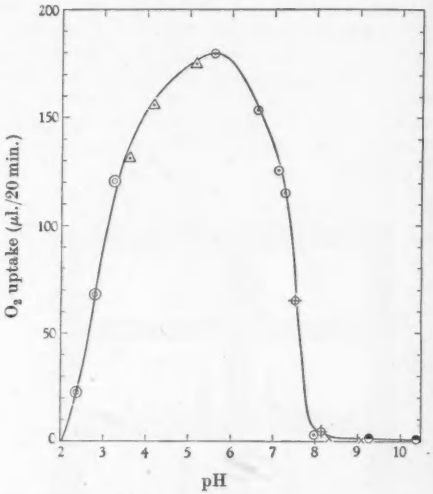

Fig. 4. Effect of pH on the activity of glucose oxidase. 15 μg. notatin, 1% glucose, 0·1 ml. catalase. 0·2M-buffers, Temp. = 39°. ◎ phosphate-citrate; ⊕ phosphate borate; △ acetate; × pyrophosphate; ⊙ phosphate; ◓ phosphate-NaOH.

and the pH's of the reaction mixtures were determined with a glass electrode. The optimum pH is 5·6 which is in close agreement with the figure given by Müller (1936) for the enzyme from *Aspergillus niger*. Variations of phosphate and acetate buffer concentrations between 0·03 and 0·25M do not influence the rates of O_2 uptake. The loss of activity above pH 8 is noteworthy and the effect of high pH was further investigated as follows:

Three borate buffers were prepared as shown in Table 1. Notatin was incubated for various periods in these buffers, and then brought to pH 5·3 by addition of the previously determined volume of 0·2M-acetate buffer pH 3·6. The activity of the enzyme was then determined under the standard conditions with glucose and catalase at pH 5·3. Table 1 gives the volumes of acetate buffer required to neutralize the borate buffers to pH 5·3. Thus in one set of experiments an aqueous solution of notatin (1·25 mg./ml.) was diluted 50 times in the mixture borate I + acetate (Table 1) and 0·5 ml. tested in presence of catalase and 1% glucose at 39°; the volume being made up to 3·3 ml. with the mixed buffer (Exp. 1, Table 2). A second sample of the aqueous notatin was similarly diluted with borate I alone. At intervals up to 40 min. 0·5 ml. was pipetted into a Barcroft flask containing 1·5 ml. borate I + 1·0 ml. acetate buffer. The enzyme was then tested with glucose and catalase as before (Exps. 2–5, Table 2).

Table 1. *Acidities of borate buffers used to test destruction of notatin at high* pH

Buffer	Constitution	pH	Volume of 0·2 M-acetate buffer pH 3·6 added to 10 ml. of the borate buffer to give final pH 5·3 (±0·1) (ml.)
Borate I	0·05 M-borax	9·1	5
Borate II	100 ml. I + 50 ml. 0·1 M-KH_2PO_4	8·1	3
Borate III	100 ml. I + 75 ml. 0·1 M-KH_2PO_4	7·6	2·5

Table 2. *Destruction of notatin by incubation at room temperature at* pH 9·1

(12·5 μg. notatin, 1% glucose, 0·1 ml. catalase.)

Exp. no.	Time of incubation at pH 9·1	Activity after neutralization to pH 5·3 (μl. O_2/25 min.)
1	0	296
2	2	25
3	7	24
4	23	7
5	40	0

Table 2 shows the rapid disappearance of enzyme activity that follows incubation at the high pH. These results are plotted in Fig. 5 (line *A*) together with similar experiments in which the incubation pH was 8·1 and 7·6. Experiments in which glucose was present during the incubation period (dashed lines) show that the substrate can protect the enzyme to a considerable extent from destruction at pH > 8.

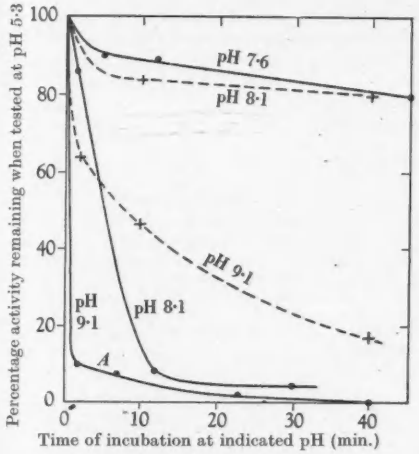

Fig. 5. Loss of activity of glucose oxidase following incubation at room temperature at pH 7·6, 8·1 and 9·1 for various periods prior to neutralization to pH 5·3 and testing with glucose at 39°. 12·5 μg. notatin, 1% glucose, 0·1 ml. catalase. •—• incubation in absence of glucose. ×—× incubation in presence of glucose.

Unless otherwise stated, all subsequent experiments were carried out in 0·2 M-phosphate buffer pH 5·6.

Effect of substrate concentration and oxygen tension at 39°. The effect of glucose concentrations was determined with 5 μg. notatin and glucose concentrations up to 7·3%. From the results obtained (Fig. 6) the Michaelis constant is found to be

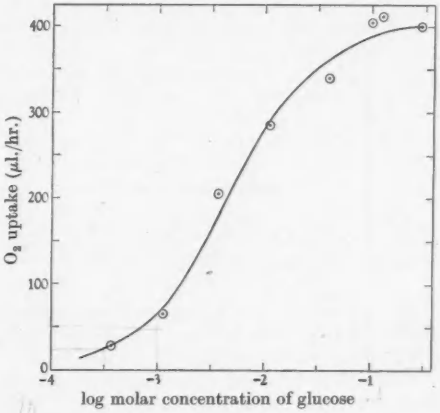

Fig. 6. Effect of substrate concentration on glucose oxidase. 5 μg. notatin, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°.

$K_m = 0{\cdot}0042$ M which is considerably lower than the value 0·026 found by Franke & Lorenz (1937) with a crude enzyme preparation.

The influence of O_2 tension is summarized in Fig. 7 from which it is seen that the activity in pure O_2 is nearly $2\frac{1}{2}$ times higher than in air. In these experiments 10 μg. notatin was used. From Fig. 6 the Q_{O_2} of the enzyme at maximum glucose concentration is found to be 82,000 while from Fig. 7 where less glucose was present the figures are 60,000 and 140,000 in air and O_2 respectively. Coulthard *et al.* estimate the Q_{O_2} of their purest enzyme to be 120,000 at 30° in O_2, a figure which is corroborated by our results. As the purity of our sample of notatin was 80–90% the activity of the completely pure enzyme may be expressed by increasing these Q_{O_2} values by about 10%. In comparing these figures with those of previous workers (e.g. Franke & Deffner (1939) reach $Q_{O_2} = 8000$) it should be remembered that the earlier experiments were carried out in O_2 at 30° in absence of catalase. The absence of catalase will tend to increase the O_2 uptake

(Keilin & Hartree, 1945*b*) while the lower temperature will slightly decrease it (see below) hence the old and new figures can be considered as reasonably comparable. Franke & Deffner's preparations were therefore only 5–10 % pure.

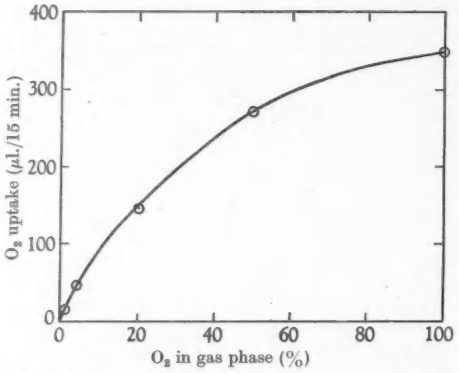

Fig. 7. Effect of O_2 tension on the activity of glucose oxidase. 10 μg. notatin, 1 % glucose, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°.

Effect of temperature. Fig. 8 shows the relationship between enzyme activity and temperature using 12·5 μg. notatin under the standard conditions. The temperature coefficient of the reaction falls off rapidly with rising temperature.

Substrate specificity. In Table 3 are shown the various sugars and sugar derivatives which have been tested with glucose oxidase. The standard conditions for the test were 0·05 M-substrate, 0·1 ml. catalase and 300 μg. notatin. For comparison purposes glucose was tested under the same conditions with 10 μg. notatin from which the O_2 uptake with

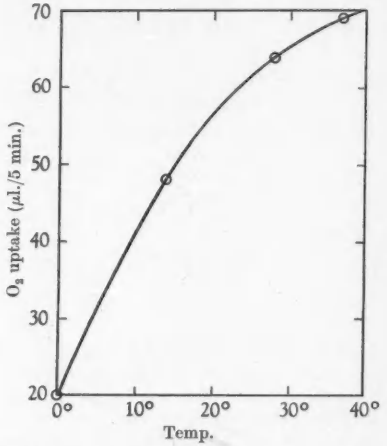

Fig. 8. Effect of temperature on the activity of glucose oxidase. 12·5 μg. notatin, 1 % glucose, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6.

Table 3. *Specificity of glucose oxidase*

Not oxidized

Sugars	Methylated sugars	Methyl glucosides
Arabinose	2-Methylglucose	α-Methylglucoside
Fructose	3-Methylglucose	2-Methyl-α-methylglucoside
Fucose	2:3-Dimethylglucose	2:3-Dimethyl-α-methylglucoside
Glucoheptose	2:3:6-Trimethylglucose	2-Methyl-β-methylglucoside
Lactose	2:4:6-Trimethylglucose	2:3:6-Trimethyl-β-methylglucoside
Melizitose	2:3:4:6-Tetramethylglucose	
Raffinose	2:4-Dimethyl-α-galactose	Acetyl glucoses
Rhamnose	4:6-Dimethyl-α-galactose	2:3:4:6-Tetraacetylglucose
Ribose	2:4:6-Trimethyl-α-galactose	Pentaacetylglucose
Sorbose	2-Methyl-β-galactose	3:4:6-Triacetyl-2-deoxyglucose
Sucrose	2:6-Dimethyl-β-galactose	
Tagatose	3:4-Dimethyl-β-galactose	Phosphorylated sugars
	3:4:6-Trimethylfructofuranose	Glucose-1-phosphate
		Hexose-6-phosphate
		Fructosediphosphate

Oxidized (glucose = 100)

6-Methylglucose	1·85	Melibiose	0·11
4:6-Dimethylglucose	1·22	Cellobiose	0·09
Mannose	0·98	Glucuronic acid	<0·05
Xylose	0·98	Galacturonic acid	<0·05
Trehalose	0·28	2-Aminoglucose	<0·05
Maltose	0·19	2-Aminogalactose	<0·05
Galactose	0·14		

300 μg. could be calculated. Although the rate of O_2 uptake is not strictly proportional to the quantity of enzyme taken, the figures obtained for the various sugars are nevertheless comparable among themselves. Franke & Deffner (1939), using an enzyme preparation of $Q_{O_2} = 2000$, give the ratio of O_2 uptakes as follows: glucose : mannose : galactose = 1 : 0·07 : 0·14. Similar ratios are recorded by Müller (1941). From Table 3 it is seen that of the commoner sugars, apart from glucose, only mannose and xylose are oxidized though at a rate $> 1\%$ that of glucose. Galactose is much less readily attacked and the previously recorded higher activities are undoubtedly due to the glucose which is invariably present in samples of galactose prepared in the normal way by hydrolysis of lactose. This point is illustrated in Fig. 9, which shows the O_2 uptake under the standard conditions with 300 μg. notatin with a commercial sample of 'pure' galactose, and a specially purified sample kindly provided by Dr D. J. Bell. With all commercial galactoses there is an initial rapid O_2 uptake followed by a slow but steady oxidation of the galactose. By extrapolation to zero time the glucose content may be estimated. Thus in the upper curve of Fig. 9 the glucose content $\equiv 80$ μl.

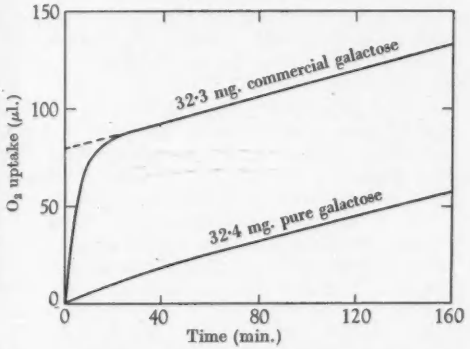

Fig. 9. Oxidation of pure galactose, and commercial galactose containing glucose, by glucose oxidase. 300 μg. oxidase, 0·1 ml. catalase. pH = 5·6, Temp. = 39°.

O_2, i.e. 1·29 mg. or 4·0 %. It is impossible to be certain that all phosphorylated sugars are unattacked as the very slight O_2 uptakes frequently observed may be due to a slow hydrolysis. The slow oxidations of 6-methyl- and 4:6-dimethylglucose, however, appear to be genuine since the rates of oxidation remain constant over long periods.

Inhibitors of glucose oxidase. Working with enzyme preparations of Q_{O_2} 20–30 Franke & Lorenz (1937) found that narcotics (alcohols, urethanes) had a marked inhibitory effect comparable in magnitude to that observed with, for example, succinic dehydrogenase. However, we have found the pure oxidase not to be affected by this class of substances. The respiratory inhibitors HCN, H_2S, HN_3 do not inhibit glucose oxidase, a fact which follows from the flavine nature of the enzyme, but may even give rise to an increased O_2 uptake. This increase is due to the poisoning of the catalase present in all but the purest samples of notatin and the consequent accumulation of H_2O_2. In his earliest experiments Müller (1928) records a slight inhibition of his enzyme by cyanide although as high a concentration as 0·5 M was required in order to obtain 50 % inhibition.

In attempts to find a powerful, specific and reversible inhibitor of glucose oxidase we have tested a large range of substances but without positive results. In a few cases a very slight inhibition was observed. Thus under the standard conditions 0·01 M concentrations of 8-hydroxyquinoline, sodium nitrate and semicarbazide gave rise to 11, 13 and 20 % inhibition respectively.

Oxidation of glucose by Penicillium notatum. The mould (strain 4222 of the National Collection of Type Cultures) was grown in plugged Barcroft manometer

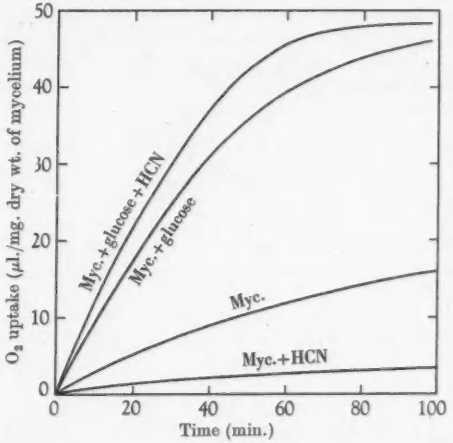

Fig. 10. Oxidation of glucose by washed 10-day mycelia (Myc.) of *Penicillium notatum*. Effect of cyanide. 2% glucose, 0·004 M-HCN, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°. The dry weights of mycelia, grown in manometer flasks, were 21–25 mg.

flasks on 5 ml. Czapek-Dox medium (Coulthard *et al.* 1945). After 3–10 days growth at 24° the medium was sucked off and replaced with successive lots of 0·2 M-phosphate buffer pH 5·6. In this way all traces of the medium were removed. The respiration

of the mycelia was tested at pH 5·6 in presence of 2% glucose and/or 0·002 M-HCN. The centre wells of the manometers contained a roll of filter paper soaked in 10% NaOH or, when cyanide was present, with the appropriate NaOH-KCN mixture (Umbreit Burris & Stauffer, 1945). The results of such experiments with a 10-day culture are shown in Fig. 10. The O_2 uptakes are given for mg. dry weight of mycelium, the dry weight of the latter (21–25 mg.) being determined at the end of the experiment. Experiments with a 3-day culture, showing a much higher Q_{O_2}, gave essentially the same results. Although the endogenous respiration is cyanide-sensitive, the oxidation of glucose is not. Hence it must be accepted that the intracellular oxidation of glucose to gluconic acid, like the extracellular reaction, does not involve the cytochrome system.

Cultivation of Penicillium notatum *on a fructose medium.* An attempt to affect the specificity of glucose oxidase was made by repeated subcultivation on a Czapek-Dox medium in which glucose had been replaced by fructose. Small plugged test tubes were used, each containing 1 ml. medium. The organism grew well at 24°, and was subcultivated every 4 days within which time a thick crop of spores was produced under these conditions. After 20 passages the culture was inoculated into two culture dishes, 15 cm. diameter, each containing 200 ml. of the fructose medium. Ten days later the yellow culture medium was removed and tested in the usual way with glucose and fructose. It was found to be still completely specific for glucose.

SUMMARY

1. Mould glucose oxidase of Müller obtained in the highly purified state (notatin) by Coulthard *et al.* from *Penicillium notatum* was investigated in respect to its structure, physical properties and enzyme kinetics.

2. The enzyme, as was previously shown, catalyzes the oxidation of glucose to gluconic acid by molecular O_2, which is reduced to H_2O_2

$$(C_6H_{12}O_6 + H_2O + O_2 = C_6H_{12}O_7 + H_2O_2).$$

3. The enzyme, which is a flavoprotein, shows in the oxidized state the characteristic absorption spectrum with bands at 377 and 455 mμ. On addition of glucose the enzyme is almost completely decolorized and the bands become hardly perceptible. They disappear completely in presence of $Na_2S_2O_4$. That the prosthetic group is the alloxazine-adenine dinucleotide was demonstrated by removing the prosthetic group from D-amino-acid oxidase, and regenerating the activity of the latter by the addition of boiled notatin.

4. From the absorption constant at 455 mμ. the minimum molecular weight is estimated to be about 75,000. The molecular weight calculated from the sedimentation velocity and diffusion constant is 152,000 (see addendum), which shows that the molecule of enzyme contains two prosthetic nuclei.

5. From the behaviour of the most active enzyme preparation in the ultracentrifuge and in the Tiselius electrophoresis apparatus, it would appear to be between 80 and 90% pure.

6. The enzyme does not fluoresce in ultraviolet light within the pH limits of its catalytic activity (pH 2–8). Outside this range it loses its activity and becomes fluorescent.

7. The enzyme shows a pronounced specificity for glucose. Of about 50 sugars and their derivatives tested, four are oxidized at about 1% of the rate of glucose oxidation (mannose, xylose, 6-methylglucose and 4:6-dimethylglucose), whereas the remainder are virtually or completely unattacked. The previously recorded oxidations of galactose are due to glucose impurities.

8. In experiments with mycelia of *P. notatum* grown in manometer flasks it was found that the endogenous respiration of the mycelium was cyanide-sensitive, but that the oxidation of glucose by the mycelia was not inhibited by cyanide. Thus the cytochrome system is not involved in the intracellular oxidation of glucose to gluconic acid.

9. Attempts to modify the specificity of glucose oxidase by repeated subcultivation on a fructose medium were unsuccessful.

The authors wish to thank Prof. H. Raistrick, Dr J. H. Birkinshaw and Dr W. F. Short for generous gifts of highly purified notatin, Dr D. J. Bell and Prof. M. Stacey for numerous samples of sugars, and Dr M. Dixon for facilities in the use of the Beckman photoelectric quartz spectrophotometer and the Tiselius electrophoresis apparatus.

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Addendum. Sedimentation and Diffusion of Glucose Oxidase (Notatin)

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(*Received* 7 *July* 1947)

A sample of glucose oxidase (Coulthard *et al.* 1945) was examined with the Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938), and its diffusion constant was measured by a new method (Coulson, Cox, Ogston & Philpot, 1948). There was a main sedimenting component (Fig. 1 (*a*)) which accounted for at least 80% of the total refracting material and which appeared to be homogeneous: in addition, there were small quantities of a faster and a slower component (Fig. 1 (*b*) and (*c*)). The whole of the absorption at 495–515 mμ. was associated with and sedimented at the same speed as the main component. The buffer was NaCl 0·2M; KH_2PO_4 0·057M; NaH_2PO_4 0·0095M: the speed of rotation was 1015 rev./sec. The results are given in the table.

The values of M and of f/f_0 are obtained assuming a value of 0·75 for the partial specific volume of the protein. The value of D_{20} is an average for all the protein present; since this is not strictly monodisperse, the value may not be accurate for the main component. However, the value of M is in reasonable agreement with that of the equivalent weight as estimated in the preceding paper.

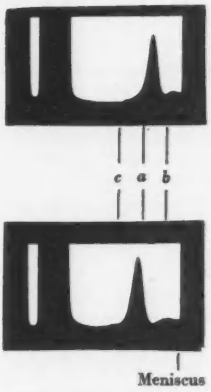

Fig. 1. Diagrams of glucose oxidase boundaries 15 and 25 min. after reaching 1015 rev./sec.

Refractive increment $\times 10^4$	Concentration (g./100 ml.)	S_{20} $\times 10^{13}$	S_{20} (corr.) $\times 10^{13}$	D_{20} $\times 10^7$	M	f/f_0
13·5	0·75	7·25	7·86	—	—	—
6·4	0·36	7·62	8·27	5·13	152,000	1·16

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The Use of Glucose Oxidase (Notatin) for the Determination of Glucose in Biological Material and for the Study of Glucose-producing Systems by Manometric Methods

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Glucose oxidase or notatin, obtained in a highly purified state from culture media of *Penicillium notatum* (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1945), catalyzes the aerobic oxidation of glucose to gluconic acid. The fact that this enzyme shows both a high affinity and a marked specificity for glucose (see preceding paper) suggested the possibility of its utilization for the estimation of glucose in presence of other sugars or of proteins, and for the study of the kinetics of different enzyme reactions which involve the liberation of glucose from its derivatives such as di- and polysaccharides, phosphorylated sugars and glucosides. Manometric methods, owing to their rapidity and simplicity, have great advantages over other methods of analysis on a micro scale and have been applied, for example, to the estimation of urea by urease, glutathione by glyoxalase, succinic acid by muscle preparations and amino-acids by specific bacterial decarboxylases. An outstanding merit of such methods is that they can be made far more specific than any purely chemical methods. The use of notatin extends the application of manometric methods to all reactions which involve the liberation of glucose.

In this paper we shall describe various examples of the use of notatin for the manometric estimation of glucose, and for manometric studies of the kinetics of reactions catalyzed by different enzymes.

EXPERIMENTAL

If the amount of glucose present in a solution is being determined, the notatin must be added from dangling tubes when the manometers have been equilibrated in the thermostat. On the other hand, in studying the rate of production of glucose by a hydrolytic enzyme either this enzyme or its substrate (e.g. a glucoside) should be added from the dangling tube in order that glucose production may be followed in terms of O_2 uptake from the beginning of the reaction. It is important in all experiments of the latter type that the notatin should be present in excess, and that the rate of production of glucose should be the limiting factor in the overall reaction. Under such conditions there will be no accumulation of glucose in the reaction mixture and the rate of O_2 uptake catalyzed by notatin will be a genuine measure of glucose production, i.e. of the activity of the hydrolytic enzyme.

The oxidation of glucose by the highly purified glucose oxidase proceeds quantitatively and the O_2 uptake under specified conditions can be taken as a measure of the glucose present (Keilin & Hartree, 1945*a*). The primary oxidation reaction is the formation of gluconic acid and H_2O_2:

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2. \quad (1)$$

When using very pure glucose oxidase (notatin) in slightly acid solution (the optimum pH is 5·6) the H_2O_2 is not decomposed at an appreciable rate, and the final value of the O_2 uptake will be 1 mol. O_2/mol. glucose (124·4 μl. O_2/mg. glucose). As a general rule part of the H_2O_2 decomposes, and in order to estimate glucose by a manometric technique it is essential to add catalase which will decompose the H_2O_2 as it is formed. The nett reaction is then

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 = C_6H_{12}O_7, \quad (2)$$

and the O_2 uptake will be 62·2 μl./mg. glucose. We have shown previously that the addition of certain alcohols to an enzyme system producing H_2O_2 will, in presence of catalase, lead to an oxidation of the alcohol to the aldehyde (Keilin & Hartree, 1936*a*, 1945*a*). This coupled oxidation of the alcohol is catalyzed by catalase. The overall reaction is now

$$C_6H_{12}O_6 + O_2 + R.CH_2OH = C_6H_{12}O_7 + H_2O + R.CHO, \quad (3)$$

and, if the alcohol is present in excess, the O_2 uptake again reaches the value of 124·4 μl./mg. glucose.

It is important in all quantitative estimations of O_2 uptake to ascertain that under the experimental conditions the H_2O_2 formed by reduction of O_2 is either quantitatively decomposed into water and O_2 or completely utilized in a coupled oxidation. Under either of these conditions there is a stoicheiometric relationship between the glucose oxidized and the O_2 uptake of the system. In absence of any substances which can undergo coupled oxidation in presence of H_2O_2 and catalase the former will be quantitatively decomposed into water and O_2, but

in practice part of the H_2O_2 will as a rule be utilized in coupled oxidations. It is therefore advisable in all cases to investigate the effect on the system of ethanol, the presence of which will ensure that any H_2O_2 not utilized for other coupled oxidations will be utilized for oxidation of ethanol to aldehyde.

Whereas in our experiments we have made use of pure glucose oxidase, satisfactory results can be obtained with crude material and even with culture media of *P. notatum* obtained as described by Coulthard *et al.* (1945). Such enzyme preparations will naturally be more liable to exhibit coupled oxidations than the pure material.

Detergents and proteolytic enzymes are sometimes used for the preparation of tissue extracts suitable for analysis, and experiments were carried out in order to determine their effect upon notatin. We have found that after 1 hr. incubation at 39° and pH 5·6 with 0·2% sodium deoxycholate, 0·2% sodium dodecylsulphate, 1% pepsin or 1% papain (in presence of cysteine) the catalytic activity of notatin was unimpaired.

Oxygen uptakes were measured in Barcroft differential manometers, usually at 39°. The flasks contained a total volume of 3·3 ml., one of the components of the system being added from a dangling tube so that the complete O_2 uptake from zero time could be recorded. Whenever possible the pH of the reaction mixture was at or near 5·6, although any pH between 3·5 and 7 is permissible. The highly purified sample of notatin (see preceding paper) was made up as a 0·1% aqueous solution which was stable for 1 week at 5°. Catalase was obtained from horse liver by the method previously described (Keilin & Hartree, 1945*b*).

Estimation of glucose in blood plasma

Samples of oxalated blood of a rat (A) or of cats (B, C, D) were centrifuged immediately after removal from the animal and each sample was tested in two manometers. The right-hand flask of one manometer received 2 ml. plasma, 0·1 ml. catalase and buffer (0·25 M-phosphate, pH 5·6) to 3·0 ml. while the second manometer received the same ingredients plus 0·1 ml. 20% (v/v) ethanol. In each flask was placed a dangling tube containing 0·3 ml. notatin solution (300 μg.). Both left-hand flasks received 3·3 ml. buffer. After equilibration in a thermostat at 39° the dangling tubes were dislodged and the O_2 uptakes followed manometrically. In presence of ethanol the H_2O_2 formed during the oxidation of glucose should be quantitatively utilized for oxidation of ethanol according to equation (3). In absence of ethanol the reaction should follow equation (2) unless there are in plasma oxidizable substances which can take the place of ethanol in coupled oxidation.

Fig. 1 shows the results obtained with plasma A. In absence of ethanol, 1 mg. glucose $\equiv$ 62·2 μl. O_2 and the final O_2 uptake corresponds to 3·63 mg. glucose. In presence of ethanol 1 mg. glucose $\equiv$ 124·4 μl. O_2 and the O_2 uptake corresponds to 3·37 mg. glucose. In all cases the experiments without ethanol gave O_2 uptakes slightly more than half that observed in presence of ethanol. In Table 1 are summarized the results obtained with the 4 samples of plasma together with figures for reducing sugar estimated by the Hagedorn & Jensen method (1923). The latter method gives results which are

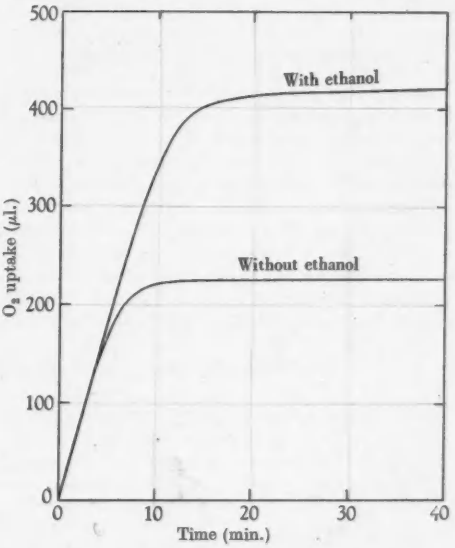

Fig. 1. Oxidation by notatin of the glucose in 2 ml. rat plasma in presence and in absence of 20 mg. ethanol. Each right-hand flask contains 300 μg. notatin, 0·1 ml. catalase, 2 ml. plasma and 0·1 M-phosphate buffer pH 5·6. Temp. = 39°.

Table 1. *Estimation of glucose in blood plasma (a) manometrically with notatin + catalase ± ethanol (see Fig. 1 for details) and (b) by the Hagedorn & Jensen method*

	Glucose found by manometric methods (mg.)		Hagedorn & Jensen estimation of glucose (mg.)		
	− Ethanol	+ Ethanol	Total reducing substances as glucose	Non-fermentable as glucose	Glucose (corrected)
2 ml. plasma A (rat)	3·63	3·37	4·05	—	—
2 ml. plasma B (cat)	4·40	4·06	4·73	—	—
2 ml. plasma C (cat)	3·20	2·72	3·45	0·63	2·82
2 ml. plasma D (cat)	3·53	2·99	3·67	0·66	3·01

higher than those obtained by either manometric method. However, in two cases a correction was made for the non-fermentable reducing substances in the plasma by estimating the apparent glucose content before and after fermenting the glucose by means of yeast. The procedure described by Mann (1946) was used for this purpose. When this correction was applied the agreement between the Hagedorn & Jensen method and the manometric method in presence of ethanol was very satisfactory.

Estimation of glucose in other biological media

The method just described for blood plasma can be applied generally to plant and animal fluids or tissue extracts. In the case of some plant extracts (e.g. potato) it is necessary to destroy the phenol-oxidases which, in presence of the natural substrates in the extracts, give rise to a rapid O_2 uptake with consequent formation of highly coloured oxidation products. The enzyme is rapidly destroyed by heating to 80° for 10–15 min., and after such treatment the O_2 uptake of the extract alone will be very small and can be applied as a correction to the uptake in presence of notatin. Tests have been carried out on cabbage sap and on press juices from potato, beetroot and mangold, and in each case the extract was tested (*a*) with notatin and catalase and (*b*) with notatin, catalase and ethanol, at 39° under the above conditions. The results are summarized in Table 2. As in the case of plasma it is seen that higher values are obtained in absence of ethanol owing to a partial utilization of H_2O_2 for coupled oxidation. The results in presence of ethanol are therefore the more reliable. By carrying out an estimation in presence of invertase, both glucose and sucrose may be determined, the difference in O_2 uptakes in presence and in absence of invertase, being a measure of the glucose equivalent of the sucrose. In such experiments the O_2 uptake reaches a steady value in 20–30 min.

By the manometric method as little as 0·1–0·2 mg. glucose may be accurately estimated. The main advantages of the method are its simplicity, the fact that it is not necessary to remove proteins and, above all, the high specificity of notatin for glucose. On the other hand the method requires about 10 times the quantity of material necessary for the Hagedorn & Jensen method. Furthermore, the manometric method cannot be used for analysis of whole blood as part of the oxyhaemoglobin will become oxidized to methaemoglobin with consequent liberation of the bound O_2. When the quantity of glucose is too small for manometric estimation notatin may be utilized in connexion with the Hagedorn & Jensen method. Thus by estimation of reducing substances by the latter method, before and after incubation with notatin, the glucose may be accurately estimated. This was done by Mann (1946) who has shown that the reducing values of seminal plasma before and after treatment with glucose oxidase were approximately the same. In this way he demonstrated the virtual absence of glucose in the seminal plasma, and confirmed his discovery that the main reducing carbohydrate of seminal plasma is D-fructose.

Table 2. *Glucose estimation in various plant extracts using notatin*

(300 μg. notatin, 0·1 ml. catalase, 0·1 M-phosphate buffer pH 5·6. Temp. = 39°. O_2 uptakes shown are the final steady values.)

System in manometer	Plant juice alone	Plant extract + notatin + catalase*		Plant extract + notatin + catalase + ethanol†		Plant extract + notatin + catalase + ethanol + invertase†	
	O_2 uptake (μl.)	O_2 uptake (μl.)	Glucose (mg.)	O_2 uptake (μl.)	Glucose (mg.)	O_2 uptake (μl.)	Sucrose (mg.)
2 ml. cabbage sap	0	286	4·60	495	3·97	—	—
1 ml. potato press juice (heated)	7	173	2·64	308	2·42	—	—
0·5 ml. beetroot press juice	3	179	2·83	323	2·57	538	3·28
0·5 ml. mangold press juice	0	245	3·94	391	3·14	1048	10·05

* 1 mg. glucose ≡ 62·2 μl. O_2 (see p. 230).

† 1 mg. glucose ≡ 124·4 μl. O_2 (see p. 230).

Studies on invertase

(*a*) *Activity of invertase* in vitro. The standard methods for the study of invertase involve the use of the polarimeter or the periodic acid estimation of reducing sugar. The former gives a continuous record, but cannot be applied to solutions which are cloudy or which contain large quantities of other optically active substances. The latter method only gives the quantity of sucrose inverted at one instant during the reaction, and becomes laborious when continuous records of a number of experiments are required. Neither of these difficulties arise when the reaction is carried out in presence of notatin and the glucose production followed by the O_2 uptake in a manometer. As a source of enzyme British Drug Houses

'invertase concentrate' was used at a dilution of 1 in 250 and the manometric method was checked by estimation of reducing sugar at the end of the experiment.

Two Barcroft manometers were filled as follows: The right-hand flask of each manometer received 0·3 ml. diluted invertase, 0·6 ml. 0·1% notatin, 0·1 ml. catalase, 2·0 ml. 0·25 M-phosphate pH 5·6 and 0·3 ml. 10% sucrose in a dangling tube. The left-hand flasks received 3·3 ml. buffer. The experiments were carried out at 20°, and the sucrose was added after equilibration and closing the manometer taps. One manometer was shaken for 28 min. from the addition of sucrose when the O_2 uptake was 312 μl. At 29 min. 1 ml. liquid from the right-hand flask was added to a hot mixture of 25 ml. 0·45% $ZnSO_4.7H_2O$ and 5 ml. 0·1 N-NaOH, heated at 100° for 3 min. and filtered. The reducing sugar was estimated in the filtrate by the Hagedorn & Jensen method. The second manometer showed an O_2 uptake of 470 μl. at 42 min. At 43 min. the reducing sugar in the left-hand flask was similarly estimated. The O_2 uptakes for 29 and 43 min. were calculated from the observed values at 28 and 42 min. respectively, and the results obtained by the two methods are shown in Table 3 where the glucose found manometrically is seen to be very close to the residual reducing sugar (fructose).

Table 3. *Invertase activity measured (a) manometrically with notatin, and (b) by estimation of reducing sugar*

(0·3 ml. invertase (B.D.H. concentrate 1 : 250), 600 μg. notatin, 0·1 ml. catalase, 30 mg. sucrose. 0·15 M-phosphate pH 5·6. Temp. = 20°.)

	In 29 min.	In 43 min.
O_2 uptake (μl.)	312	470
Glucose equivalent (mg.)	5·01	7·57
Residual reducing sugar = fructose (mg.) by Hagedorn & Jensen method	5·13	7·40

In Fig. 2 is shown the effect of varying concentrations of sucrose on the activity of an invertase solution at 17° and pH 5·6. Nelson & Sottery (1924) have shown that low concentrations of glucose activate, and higher concentrations inhibit the initial velocity of hydrolysis of sucrose by honey-bee salivary invertase. The rate of inversion was, on the other hand, only slightly affected by fructose. The effect of high concentrations of fructose or invertase could not be readily determined by the usual methods, but by means of notatin it could be shown manometrically that 0·167 M-fructose inhibits the rate of hydrolysis of 0·007 M-sucrose in presence of invertase by only 18%. The study of the kinetics of the invertase reaction by means of notatin is simplified owing to the complete elimination of glucose which may either accelerate or retard the reaction.

(*b*) *Activity of invertase* in vivo. Yeasts contain an exceptionally powerful invertase which, from our preliminary experiments, appeared to be far more active than would be necessary to maintain a sufficient supply of monosaccharide for normal metabolic requirements. It therefore seemed probable that in presence of sucrose, respiring yeast cells would discharge large quantities of invert sugar into the surrounding medium. The yeast selected for this

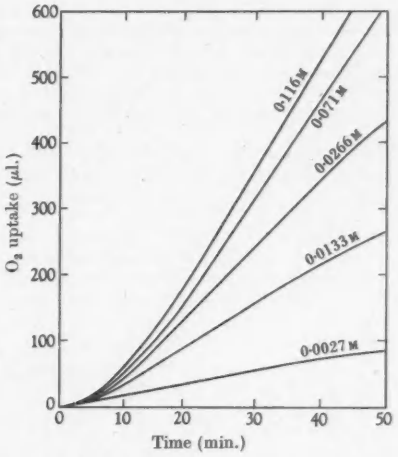

Fig. 2. Inversion of varying concentrations of sucrose by invertase followed manometrically by oxidation of the liberated glucose by notatin. Each right-hand flask contains 0·2 ml. invertase concentrate (1 : 250), 500 μg. notatin, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Figures express concentration of sucrose. Temp. = 17°.

work was *Torulopsis utilis* (National Collection of Type Cultures no. 2576). This yeast does not ferment sugars under aerobic conditions, and it therefore becomes a simple matter to compare its metabolic requirements with the activity of the intracellular invertase by a simple manometric technique using notatin.

The organism was grown in Roux flasks on beer wort containing 3% agar and adjusted to pH 6·5. The flasks containing the medium were sterilized for 20 min. at 100° on three successive days. After inoculation the flasks were incubated at 30° for 2 days and then at room temperature for 3 days. The cells were collected in distilled water and washed repeatedly with distilled water in centrifuge tubes. A suspension in water was prepared containing 0·2 mg. dry wt./ml. Table 4 shows the ingredients of the right-hand flasks of the four manometers used for the test. The left-hand flasks received 3·0 ml. buffer and a roll of filter paper soaked in 10% NaOH.

In view of the very high invertase activity as little as 0·1 mg. dry weight of the yeast is sufficient. The Q_{O_2} was estimated separately on a larger quantity of yeast (Fig. 3). After closing the taps the manometers were shaken for 15 min. prior to addition of sucrose from the dangling tubes: during this period the O_2 uptake was negligible. At

15 min. the sucrose was added and the O_2 uptake followed for the next 55 min. (Fig. 3). At this point the reaction was stopped by addition to each right-hand flask of a hot mixture of 1 ml. 4·5% $ZnSO_4.7H_2O$, 0·2 ml. N-NaOH and 6 ml. H_2O. The quantities of reducing sugars, estimated as already described, in the protein-free filtrates, are given in Table 4 together with the corresponding O_2 uptakes. The residual reducing sugar in manometer 1 = invert sugar formed minus sugar oxidized by yeast, and this value must be corrected by the figure from manometer 4 which represents the reducing value of the yeast. Similarly the reducing sugar formed in manometer 2 represents sugar inverted minus sugar oxidized by yeast and notatin, and this figure must be similarly corrected from manometer 3.

Table 4. *Invertase activity of* Torulopsis utilis *determined (a) manometrically with notatin, and (b) by estimation of reducing sugars in presence and in absence of notatin*

(The volumes in the right-hand flasks of the manometers were made up to 3 ml. with phosphate buffer (0·25 M, pH 5·6) and the central tube received 0·3 ml. 10% NaOH and a roll of filter paper. The left-hand flasks received 3 ml. buffer and a roll of filter paper soaked in 10% NaOH.)

Components of system (right-hand flask)	Manometer no. 1	2	3	4
0·5 ml. *T. utilis* (0·1 mg.)	+	+	+	+
300 μg. notatin + 0·1 ml. catalase	−	+	+	−
0·3 ml. 10% sucrose in dangling tube	+	+	−	−
O_2 uptake in 55 min. (μl.)	9	250	0	0
Total reducing sugar at 55 min. (mg.)	8·63	4·83	0·02	0·02

Hence glucose oxidized = (1 − 4) − (2 − 3) = 3·80 mg. From the O_2 uptake in manometer 2 the glucose content is 250/62·2 = 4·01 mg. A correction must be applied for the slow oxidation of inverted sucrose (9 μl. O_2) detected in manometer 1. The complete oxidation of 1 mol. invert sugar by yeast requires 12 atoms oxygen instead of the 1 atom utilized by notatin. The correction to be applied to the O_2 uptake in manometer 2 is therefore $9/x$ where x may be as high as 12. The correction is within experimental error, and the glucose oxidized according to the manometric method may be taken as 4·00 mg. This value may be too high for two reasons: (*a*) The sucrose used in these experiments showed a slight reducing action in the Hagedorn-Jensen estimation (= 0·06 mg./manometer) and part at least of this impurity may be glucose, (*b*) a slight coupled oxidation of substances in the yeast or the sucrose may take place (as in the case of the estimation of glucose in plasma (Table 1)). The agreement between the two methods is therefore satisfactory.

From the above results it is seen that 0·1 mg. *T. utilis* produces 2 × 3·8 mg. invert sugar in 55 min., i.e. 1 mg. produces 83 mg./hr. The complete oxidation of this quantity of sugar by the organism would require 83 × 62·2 × 12 = 62,000 μl. O_2. In other words if the yeast could oxidize the monosaccharides to CO_2 and H_2O as rapidly as they were produced its Q_{O_2} would be 62,000 instead of 112, the figure obtained experimentally (Fig. 3). There is thus a considerable 'overflow' of invert sugar.

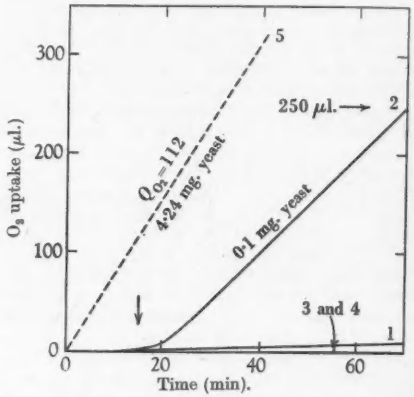

Fig. 3. Inversion of sucrose by yeast (*Torulopsis utilis*) followed manometrically in terms of oxidation of the liberated glucose by notatin. Experiment carried out at 39° in four manometers (1–4, see Table 4). Each manometer receives 0·1 mg. (dry wt.) *T. utilis* in 0·1 M-phosphate buffer pH 5·6; manometers 2 and 3 also contain 300 μg. notatin and 0·1 ml. catalase. 0·3 ml. 10% sucrose was added after 15 min. to manometers 1 and 2. Manometer 5: O_2 uptake by 4·24 mg. yeast in presence of 0·3 ml. 10% sucrose. The central tubes of all flasks receive 0·3 ml. 10% NaOH and a roll of filter paper. The numbering of the curves corresponds to that of the manometers.

Experiments similar to those just described have been carried out on baker's yeast, brewer's yeast, *B. subtilis* and mycelia of *P. notatum* (see preceding paper). The first 3 organisms showed a similar large 'overflow' of invert sugar, but *P. notatum* was unable to invert sucrose.

Maltase

The study of maltase activity by standard methods is straightforward since only 2 sugars are present in the reaction mixture, viz. maltose and glucose. Nevertheless the activity of the enzyme is most conveniently studied with the help of notatin. As a source of maltase we have used a Lebedew juice prepared by dispersing 50 g. air-dried brewer's yeast in 150 ml. water, incubating 3 hr. at 39° and filtering. In presence of catalase and 300 μg. notatin, 0·5 ml. Lebedew juice showed, under the experimental conditions already described for invertase, a very slow O_2 uptake (40 μl./hr.). On addition of maltose O_2 uptakes up to 1500 μl./hr. were observed. It was of interest to find that the maltase activity of living brewer's yeast was insufficient to provide any 'overflow' of glucose which could be detected by

means of notatin when the yeast was shaken in a manometer with maltose. The invertase activity of the brewer's yeast, however, was such as to provide a very large 'overflow' (see above).

Amylases

These hydrolyzing enzymes may be divided into two classes: the α-amylases present in pancreas, saliva, micro-organisms and malt, which break down starch into dextrins of mol. wt. 1000–3000 and finally into maltose and glucose, and the β-amylases of ungerminated seed grains which yield dextrins and β-maltose but no glucose (Hopkins, 1946). The formation of glucose and maltose from starch by

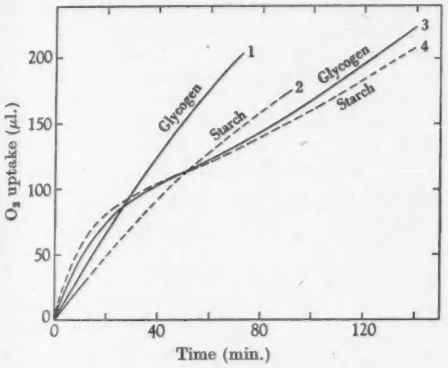

Fig. 4. Oxidation by notatin of the glucose produced from starch and glycogen by saliva and by salivary amylase. Temp. = 39°. All manometers contain 300 μg. notatin, 0·1 ml. catalase and 50 mg. polysaccharide in 0·1 M-phosphate pH 5·6. Manometers 1 and 2 contain 0·5 ml. saliva; manometers 3 and 4 contain 6 mg. amylase. The numbering of the curves corresponds to that of the manometers.

α-amylases has been studied by Somogyi (1938, 1940) and by Myrbäck (1941, 1942). While the latter has found evidence that malt amylase produces the two sugars simultaneously, the former considers that maltose precedes glucose in the reaction catalyzed by α-amylases from animal sources, and that the final quantities of maltose and glucose produced correspond to about 60 and 10% respectively of the total starch. Such investigations are hampered by the complexity of the mixtures of breakdown products and by difficulties in the determination of glucose and maltose in mixtures. The preferential fermentation of glucose by baker's yeast at pH 8·4, though far from satisfactory, has until now been the only method of determining glucose in presence of maltose. The value of notatin in such investigations is obvious and we have been able to demonstrate manometrically the formation of glucose not only from soluble starch, but also from glycogen using both saliva and an α-amylase isolated from saliva by the method of Hanes & Cattle (1938). The manometer flasks contained 300 μg. notatin, 0·1 ml. catalase, 50 mg. carbohydrate and a suitable amount of amylase. The final concentration of phosphate buffer (pH 5·6) was 0·1 M and the experiments were carried out at 39°. The O_2 uptakes are plotted in Fig. 4 where curves 1 and 2 show the formation of glucose in presence of 0·5 ml. saliva from glycogen and soluble starch respectively. Curves 3 and 4 represent corresponding experiments with 6 mg. amylase. In both cases there was no O_2 uptake in presence of maltase after a correction had been applied for the slow rate of oxidation of maltose (see preceding paper). Furthermore the polysaccharides themselves were shown to be free from glucose. These experiments offer proof of the accepted view that glucose arises from the splitting of α-1, 4 linkages in the polysaccharides and not from hydrolysis of maltose. At the same time they demonstrate a method for the study of the kinetics of glucose production by amylases; a problem to which there has hitherto been no satisfactory experimental approach. The similarity in the course of the reactions with the two polysaccharides is very striking.

Hydrolysis of glucose phosphates

The phosphatases which hydrolyze glucose-1-phosphate (Cori ester) and glucose-6-phosphate (Robison ester) are widely distributed in animal tissue. A convenient source of enzyme is the intestinal mucosa and a crude preparation was obtained as follows: the small intestine of a rabbit was well irrigated with tap water, the mucosa scraped off and ground in a mortar with an equal volume of acetate buffer (0·1 M, pH 5·6) and sand. The suspension was decanted from the sand after standing 5 min. The standard method of studying the enzyme hydrolysis of these esters is to determine at intervals the increase in inorganic phosphorus. On addition of notatin to the system, however, a continuous manometric record of glucose production can readily be obtained.

For the study of the Robison ester three manometers were filled as shown in Table 5, the notatin being dissolved in 0·3 ml. water and placed in a dangling tube. After equilibration and closing the taps, the notatin was added. The course of the reaction is shown in Fig. 5 (full lines). At 30 min. the manometer readings were taken, giving the O_2 uptakes shown in Table 5, and the reactions stopped by the addition of 3 ml. 20% trichloroacetic acid to each right-hand flask. After standing 1 hr. the mixtures were filtered and the filtrate and washings made up to 25 ml. Inorganic P was estimated by the Fiske-Subbarow method (Rona, 1929). The

increase in inorganic P due to phosphatase action is the P content of manometer 1 less that of 2 and 3, i.e. $778-32-154=592$ μg. P. The O_2 equivalent of the liberated P is similarly, from Table 5, $221-13-0=208$ μl. which corresponds $31\times208/11{\cdot}2=577$ μg. P. The figures obtained by the two methods are in good agreement.

Table 5. *Hydrolysis of Robison ester (glucose-6-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up to 3·3 ml. with 0·2 M-acetate buffer pH 5·6.)

Components of system (right-hand flask)	Manometer no. 1	2	3
1 ml. mucosa	+	−	+
300 μg. notatin + 0·1 ml. catalase	+	+	−
1 ml. Robison ester (0·1 M)	+	+	−
O_2 uptake in 30 min. (μl.)	221	0	13
Inorganic P at 30 min. (μg.)	778	32	154

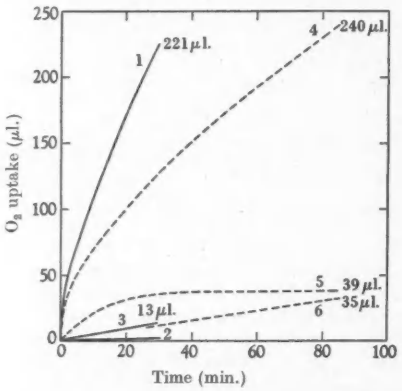

Fig. 5. Rates of hydrolysis at 39° of Robison ester (——) and of Cori ester (– – – –) catalyzed by a phosphatase preparation from intestinal mucosa. Glucose production followed by means of O_2 uptakes in presence of notatin. 0·1 M-acetate buffer pH 5·6. Curves 1 and 4: phosphatase, notatin, catalase and ester. Curves 2 and 5: notatin, catalase and ester. Curves 3 and 6: phosphatase only. For quantities see Tables 5 and 6.

A parallel investigation of the hydrolysis of Cori ester gave the results summarized in Table 6 and Fig. 5 (dashed lines). The hydrolysis of this ester is slower and the sample contained a considerable quantity of inorganic P. The results obtained after allowing the reactions to proceed for 85 min. are as follows: increase in inorganic P = $1298-363-493=442$ μg. P. Glucose liberated ≡ $240-39-35=166$ μl. $O_2\equiv460$ μg. P. In view of the large blanks the agreement is most satisfactory.

Table 6. *Hydrolysis of Cori ester (glucose-1-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up to 3·3 ml. with 0·2 M-acetate buffer pH 5·6. Temp. = 39°.)

Components of system	Manometer no. 1	2	3
1 ml. mucosa	+	−	+
300 μg. notatin + 1 ml. catalase	+	+	−
1 ml. Cori ester (0·1 M)	+	+	−
O_2 uptake in 85 min. (μl.)	240	39	35
Inorganic P at 85 min. (μg.)	1298	363	493

The initial very rapid O_2 uptakes in manometer 1 in each set of experiments is due to the accumulation of glucose during the interval required for equilibration. A more exact study of the kinetics of the reactions would require that either the phosphatase preparation or the ester be added from the dangling tube (see Introduction). The control experiment phosphatase + notatin indicated that the former contained no appreciable amount of glucose.

Activity of glucosidases

(1) *Emulsin* catalyzes the hydrolysis of amygdalin or mandelonitrile gentiobioside, the reaction proceeding as follows:

$$C_6H_5C(OH)CN.C_{12}H_{21}O_{10}+2H_2O = C_6H_5CH(OH)CN+2C_6H_{12}O_6,$$ (glucose)

$$C_6H_5CH(OH)CN = C_6H_5CHO+HCN.$$

The first stage is usually determined in a polarimeter, but is more readily followed with the use of notatin in the manner already described. The HCN produced in the second stage will, however, inactivate the catalase which is an essential component of the notatin system if a quantitative measure of glucose production is required. The most convenient way to overcome this difficulty is to add methaemoglobin which has a high affinity for HCN and which forms a stable CN-compound (Keilin & Hartree, 1936*b*) so protecting the catalase from inhibition. By this method curves 1 and 2 of Fig. 6 were obtained with 5 and 2·5 mg. emulsin respectively at 22° and pH 5·6. Each right-hand manometer flask received 2 ml. 10% crystalline methaemoglobin solution (for preparation see Keilin & Hartree, 1935) in addition to emulsin, amygdalin, notatin and catalase, and after taking readings for 45 min. the reaction mixtures were examined with a microspectroscope. At acid pH methaemoglobin shows four absorption bands in the visible region, the most prominent band being in the red at 632 mμ., while its CN′ derivative shows one broad band in the green. In both cases it was readily seen that only part of the methaemoglobin was combined with HCN. One molecule of methaemoglobin (mol. wt. 68,000) contains 4 Fe atoms and

will combine with 4 molecules of HCN. The glucose produced in Exp. 1 (Fig. 6) can be determined from the O_2 uptake, and if it is assumed that the two phases of hydrolysis of amygdalin take place simultaneously then 2 molecules of glucose will appear for each molecule of HCN. On these grounds it can be calculated that if the O_2 uptake is 400 μl., the HCN produced will be sufficient to combine with 300 mg. methaemoglobin, i.e. 3 ml. 10% solution. Since in Exp. 1 the HCN liberated is less than that required to react with 2 ml. 10% solution, it follows that the second phase of the hydrolysis is slower than the first. The second phase can best be followed by the microspectroscopic technique already described by us using a double-wedge trough, and periodically matching the absorption spectrum of the reaction mixture with that of optical mixtures of methaemoglobin, and its CN′ derivative contained separately in the two compartments of the double-wedge trough (Keilin & Hartree, 1946).

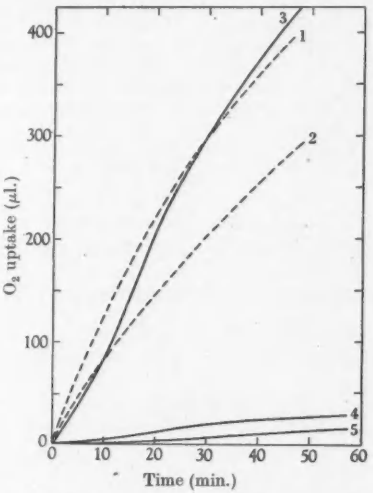

Fig. 6. (*a*) Hydrolysis of amygdalin by emulsin followed in terms of the oxidation of liberated glucose by notatin. Curve 1: 5 mg. emulsin, 60 mg. amygdalin, 2 ml. 10% methaemoglobin, 300 μg. notatin, 0·1 ml. catalase. 0·1 M-acetate buffer. Temp. = 22°. Curve 2: as 1 with 2·5 mg. emulsin. (*b*) Hydrolysis of sinigrin by sinigrinase. Glucose production followed by O_2 uptake in presence of notatin. Curve 3: 1 ml. sinigrin, 1 ml. sinigrinase, 0·1 ml. catalase, 300 μg. notatin, 0·1 M-phosphate buffer pH 5·6. Temp. = 22°. Curve 4: as 3 without notatin. Curve 5: as 3 without sinigrinase.

(2) *Emulsin* also catalyzes the hydrolysis of salicin to saligenin and glucose, and the reaction can be followed in the same way although here the methaemoglobin can be omitted.

(3) *Sinigrinase* was characterized by Neuberg & Wagner (1926) as the enzyme hydrolyzing sinigrin, a glucoside present in horse-radish root and mustard seed, the reaction proceeding as follows:

$$C_3H_5NC(OSO_3K)S.C_6H_{11}O_5 + H_2O = C_3H_5NCS + C_6H_{12}O_6 + KHSO_4.$$

allyl mustard oil

The usual manometric technique with notatin is well suited to the study of the kinetics of this system. Crude sinigrin was prepared by boiling 100 g. crushed white mustard seed with 300 ml. H_2O for 15 min. and filtering with the aid of Hyflo Supercel (Johns-Manville, London). The clear, deep-yellow filtrate was not further purified. Sinigrinase was obtained by the method of Neuberg & Wagner from the mustard seed and its reaction with sinigrin in presence of notatin is shown in Fig. 6 (curves 3, 4, 5).

SUMMARY

1. Glucose oxidase or notatin which catalyzes the aerobic oxidation of glucose to gluconic acid, shows a marked specificity and a high affinity for glucose.

2. The stoicheiometric relationship between the glucose oxidized and the oxygen utilized makes possible an accurate manometric estimation of glucose in biological fluids containing other carbohydrates and proteins.

3. The kinetics of different enzyme reactions which yield glucose can be very easily studied manometrically by means of notatin. In this way a continuous record of the hydrolytic process can be obtained in terms of O_2 uptake.

4. The use of notatin has been applied to the manometric study of the reactions catalyzed by invertase *in vitro* and *in vivo*, maltase, salivary amylase, phosphatase, emulsin and sinigrinase.

5. The use of notatin provides a method for the quantitative study of the liberation of glucose from starch and glycogen by α-amylase. It has made possible the direct identification of glucose in such reactions.

6. By means of notatin it can be determined manometrically that living yeast cells (*T. utilis*) invert sucrose 500 times faster than is necessary to provide the monosaccharides necessary for normal respiratory requirements.

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Filter-paper Partition Chromatography of Sugars

1. GENERAL DESCRIPTION AND APPLICATION TO THE QUALITATIVE ANALYSIS OF SUGARS IN APPLE JUICE, EGG WHITE AND FOETAL BLOOD OF SHEEP

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The development by Consden, Gordon & Martin (1944) of a partition method of chromatography, in which sheets or strips of filter paper are used instead of the familiar packed column, has already shown its usefulness as a method for the qualitative analysis of protein or peptide hydrolysates for amino-acids, especially in circumstances where the amount of material available is small. Although the method is not suitable for obtaining accurate quantitative results, it has the special advantage that use can be made of very small differences in partition coefficient, with the result that useful separations may be obtained with as many as nineteen or twenty amino-acids on the same chromatogram. The present communication describes an extension of the method to the qualitative analysis of sugars, either in biological fluids or in the hydrolysis products of polysaccharides. Preliminary results have already appeared (Partridge, 1946).

The essentials of the method are as follows: A small quantity of the solution to be analyzed is introduced in the form of a circular spot near the top of a sheet or strip of filter paper. The paper is then hung vertically from a trough containing a solvent which has previously been saturated with water, so that the top edge of the paper is immersed in the solvent. The trough and the filter-paper strips are then suspended inside a closed vessel, the atmosphere in which is kept in a condition of saturation both with respect to water and to the solvent. A sharp horizontal liquid front forms, which advances down the paper at a speed dependent upon the physical properties of the solvent used. To irrigate a paper strip some 35 cm. long, 18–24 hr. are usually sufficient, after which the strip may be dried and the new positions of the components of the solution rendered visible by spraying the paper with an appropriate reagent.

Under these conditions, partition of the solute takes place between water bound by the cellulose and the solvent which is moving over the surface of the cellulose fibres. The distance moved by any solute in the direction of flow is then a function of (1) the partition coefficient of the solute between water and the wet solvent, (2) the volume of water bound by unit area of the filter paper, and (3) the volume of wet solvent held by unit area of the filter paper after irrigation. In any single experiment, factors (2) and (3) are substantially constant provided the filter paper is uniform in texture, thickness and water content, so that under ideal conditions the relative distance (R_F) moved by each

solute is dependent upon its partition coefficient only.

The theory of the method has been dealt with in some detail by Consden *et al.* (1944), who give the definition

$$R_F = \frac{\text{distance moved by solute}}{\text{distance moved by advancing front of liquid}}.$$

These authors have shown experimentally that the values of R_F given by amino-acids on cellulose may be correlated fairly accurately with their partition coefficients as measured by direct methods, and the conclusion is drawn that true adsorption by the cellulose does not play a significant part.

In the work described below the reagent used to reveal the position of the spots formed by the reducing sugars has been almost exclusively a solution containing silver nitrate and a large excess of ammonia. This was sprayed on to the chromatogram after removal of the solvent used to irrigate it, and the filter-paper strip was then heated. In this way metallic silver was precipitated in the region occupied by reducing sugars, giving rise to brown or black spots. The $AgNO_3$-ammonia reagent has the advantage of being effective for all reducing sugars, and is therefore of general application to these, but for the non-reducing disaccharides or trisaccharides, other methods must be employed. For those containing a ketose, an acidic spraying reagent based on naphthoresorcinol is suitable, and details of its use are given in the text. In specialized cases other reagents were found to be of advantage, a particular instance being the use of *p*-dimethylaminobenzaldehyde as a reagent for the *N*-acetylhexosamines.

EXPERIMENTAL

With certain minor modifications, the experimental conditions that allow of separation of the amino-acids are also suitable for the qualitative analysis of sugars. Whatman no. 1 filter paper has been used exclusively, and for preparing strip chromatograms, the paper was cut into pieces 43 cm. long × about 12 cm. wide. The sugar solutions used were roughly 1% (w/v) with respect to each individual sugar, and 3–4 μl. containing 30–40 μg. of each sugar was introduced as a circular spot on a horizontal line ruled 7·5 cm. from the top of the paper strip. When a number of different sugar solutions was run on the same strip of paper, the spots were introduced at intervals of 1·5 cm. along the horizontal line. The strips were usually irrigated with the solvent for 18 hr. overnight. The solvent was dried off in an oven at 100–105°, the position of the solvent boundary being marked in ink before transfer to the oven. In order to reveal the position of the sugars the paper was sprayed rapidly and evenly with a mixture containing equal parts of silver nitrate solution (0·1 N) and ammonia solution (5 N), and the strip replaced in the oven (105°) for 5–10 min.; the sugars appeared as dark brown spots on a white or light brown ground. In runs carried out in phenol the lower part of the paper was usually uniformly coloured light brown, the upper part being white or light grey. The developed strips began to darken owing to the presence of silver salts a few hours after they had been removed from the oven, but if a permanent record was desired, the strips were washed first in distilled water and afterwards in running tap water for about 1 hr., followed by drying.

The apparatus used was that described by Consden *et al.* (1944). It was found, however, that the glass troughs could be replaced with advantage by troughs of a similar pattern made from stainless steel. For drying off the solvent and heating the papers a special oven was constructed. This had a large glass window in the front in order to allow the development of the spots to be kept under observation as the temperature increased after introducing a sprayed chromatogram. This feature was of value, since when using $AgNO_3$-ammonia as a spraying reagent it was found that certain sugars (such as glucosamine and chondrosamine) react at lower temperatures, and this effect often served as a useful confirmation of an identification based on R_F value. The oven was equipped with a small centrifugal fan which caused air heated by a bank of electrical strip heaters to be drawn slowly through the drying chamber and then to be discharged outside the building.

Use of naphthoresorcinol as a reagent for di- and trisaccharides containing ketoses

For many non-reducing oligosaccharides or glycosides the use of an acidic reagent is necessary in order to secure hydrolysis. It was found that after spraying a filter-paper chromatogram with a dilute solution of an acid and heating to 80–100°, concentration of the acid took place to a point at which dehydration of the sugars occurred, with consequent liberation of substituted furfurals. The position of the sugars could therefore be revealed by making use of the well known colour reactions of furfurals with phenolic reagents. Under certain conditions the reaction was found to be highly specific for the ketoses. A number of phenolic substances were tested for suitability as spraying reagents, and of those examined naphthoresorcinol appeared most satisfactory, although other common substances of this type, such as α-naphthol or resorcinol, were also effective. The reaction took place with phenol itself, and in chromatograms irrigated with this solvent there was usually sufficient phenol left on the paper to develop a colour with fructose on spraying with an acid and heating in the oven. Collidine inhibited the reaction to some extent, and this solvent was avoided where phenolic reagents were to be used.

A spraying reagent based on hydrochloric acid (0·25 N) and containing naphthoresorcinol (0·1% w/v) was found to be effective in producing strong red colours with the ketoses at 100–105°, but since the mineral acid tended to break down the cellulose of the filter paper it was found preferable to replace it by trichloroacetic acid. The reagent finally adopted was as follows: *Solution* (*a*) naphthoresorcinol, 0·2% (w/v) in ethanol; *solution* (*b*) trichloroacetic acid, 2·0% (w/v) in water. Equal volumes of the two solutions were mixed immediately before use, and the paper sprayed evenly with the mixture. The paper was then allowed to dry off partially at room temperature before being introduced into the oven at 100–105° for 5–10 min. Fructose, sorbose, sucrose and raffinose gave very strong red spots, the colour of which was stable for at least 12 hr.

Under the conditions described, the reaction was very selective for the ketoses, other sugars giving no more than a trace of colour at temperatures above 100°. However, on

allowing the heated chromatograms to stand for a few hours in the open air, the pentoses and uronic acids developed strong blue colorations. The presence of moisture appeared to be necessary for the development of the blue colour, and in cases where it was desired to reveal the positions of the pentoses and hexuronic acids the sprayed chromatogram was heated in a humid atmosphere at 70–80°. For this purpose, open baths of water were inserted in the oven above the heaters. At 70–80° in humid air, development of the deep blue or purple colours required 10–15 min., and under these conditions the colour due to the ketoses was orange-brown. The blue or purple colour was very stable, and was particularly intense in the case of the hexuronic acids.

Use of the Morgan & Elson reaction for hexosamine

The reagents devised by Elson & Morgan (1933) and Morgan & Elson (1934) for the colorimetric estimation of hexosamines and *N*-acetylhexosamines were adapted for use with the filter-paper technique as a second method of revealing the presence of the amino sugars. The method is of particular value for *N*-acetylglucosamine, since this substance is only slowly oxidized by the ammoniacal silver nitrate reagent, but gives an excellent reaction with *p*-dimethylaminobenzaldehyde.

The reagents were as follows:

(*a*) *Acetylacetone reagent.* Solution (1): Acetylacetone (0·5 ml.) dissolved in butanol (50 ml.). Solution (2): 50% (w/v) aqueous KOH (5 ml.) and ethanol (20 ml.). 0·5 ml. of solution (2) were added to 10 ml. of solution (1) just before the reagent was required. Crystals occasionally appeared in the mixed solution, but they readily redissolved on addition of a few drops of 50% (v/v) aqueous ethanol. The reagent was not stable and was made up daily from fresh acetylacetone.

(*b*) *p-Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (1 g.) after recrystallization from aqueous ethanol was dissolved in ethanol (30 ml.) and 30 ml. of conc. HCl added. The solution was then diluted with 180 ml. of redistilled butanol. The reagent was stable in ethanol-HCl solution and a stock solution in the solvent could be stored for several weeks.

After evaporating off the solvent, the chromatograms were sprayed with solution (*a*) and heated in the oven for 5 min. at 105°. The dry paper strips were then sprayed with solution (*b*) and returned to the oven for a further short heat treatment (5 min.) at 90°. Under these conditions the free hexosamines gave cherry-red colorations which were stable for several days. *N*-Acetylglucosamine gave a strong purple-violet colour which was also stable. However, unlike the free hexosamines, the *N*-acetyl derivatives gave strong violet colorations with the *p*-dimethylaminobenzaldehyde reagent alone, without any previous treatment with acetylacetone, and this reaction could be utilized to serve as a confirmatory test for the presence of the *N*-acetylated derivatives.

Other reducing sugars also gave colours with these reagents under the conditions described above, but the colorations were usually very light and faded rapidly, particularly on raising the oven temperature. The colours given by various neutral reducing sugars ranged from blue to pink-violet, each sugar giving a characteristic shade, but it was considered doubtful if the method would be of value for revealing the presence of the neutral sugars since the reaction was not sufficiently sensitive.

Solvents

Phenol. It was found convenient to prepare a stock solution containing phenol (detached crystals, B.P.) (900 g.) and water (100 g.). This mixture remained liquid at room temperature, and to prepare a saturated solution, the stock solution was gently shaken with an excess of water, and after separation the lower phenol layer was run off into the trough of the chromatographic chamber. The aqueous layer was run into the bottom of the chamber in order to maintain saturated conditions in the air above it. Some batches of phenol were not suitable for use as purchased, since they gave rise to a heavy black discoloration with the $AgNO_3$-ammonia reagent. Such samples could be improved considerably by steam distillation, but in one case this treatment was insufficient and the sample was further treated by shaking at intervals for several days in a large bottle with 1% (w/v) ammonia solution until the mixture acquired a deep blue colour. After steam distillation the solvent was then found to be satisfactory.

When amino sugars were under investigation, it was necessary to carry out irrigation with phenol in an atmosphere containing ammonia, since otherwise these basic sugars gave rise to elliptical spots or trails. For this purpose, the aqueous layer in the bottom of the chamber was brought to a known concentration with NH_3 solution. However, when ammonia was used a few crystals of KCN were also added to the aqueous liquid, since catalytic oxidation processes take place under the influence of traces of copper (Consden *et al.* 1944), and, in the absence of a small concentration of HCN, the advancing phenol front on the paper becomes deeply stained with dark-coloured oxidation products.

s-Collidine. This solvent was purchased as a dark-coloured liquid. Bromine (5–10 ml.) was cautiously added to the solvent (1 l.) in a large unstoppered flask with frequent vigorous shaking; after removal of the precipitate by filtration crushed sodium thiosulphate was added with further shaking, and the liquid was again filtered. The solvent was then allowed to stand 24 hr. over solid NaOH and after filtration was distilled, a middle fraction boiling at 172° being retained. Prepared in this way, the liquid remained colourless for several weeks, and after saturation with water at room temperature was used for developing the chromatograms without further additions. (This method of purification was communicated privately by Dr A. J. P. Martin.)

n-Butanol-acetic acid mixture. The butanol was purified by distillation and the dry solvent (40 ml.) was mixed with glacial acetic acid (10 ml.) and water (50 ml.). After shaking the mixture, the aqueous layer was run into the bottom of a dry chamber, and the solvent layer used to fill the trough. Since the mixture is a three-component system the volume of water contained in it is important.

RESULTS

Table 1 shows the R_F values obtained with a number of neutral sugars in certain of the more suitable solvents and solvent mixtures. In general, it has been found that only those solvents that give rise to two phases with water lead to useful separations, and further that R_F values are generally higher the

Table 1. *R_F values of neutral sugars in various solvents on Whatman no. 1 paper (corrected to 20°)*

Solvent ...	Phenol	*s*-Collidine	*n*-Butanol, 40%* Acetic acid, 10% Water, 50%	*n*-Butanol, 45%* Ethanol, 5% Water, 49%	*n*-Butanol	*iso*Butyric acid	Methyl ethyl ketone
Additions	NH_3 (1% w/v), HCN	None	None	NH_3 (1% w/v)	NH_3 (1% w/v)	None	NH_3 (1% w/v)
D-Glucose	0·39	0·39	0·18	0·105	0·070	0·13	0·025
D-Galactose	0·44	0·34	0·16	0·090	0·060	0·14	0·015
D-Mannose	0·45	0·46	0·20	0·130	0·100	0·15	0·050
L-Sorbose	0·42	0·40	0·20	0·120	0·085	0·16	0·050
D-Fructose	0·51	0·42	0·23	0·135	0·100	0·18	0·045
D-Xylose	0·44	0·50	0·28	0·170	0·125	0·19	0·090
D-Arabinose	0·54	0·43	0·21	0·145	0·100	0·21	0·075
D-Ribose	0·59	0·56	0·31	0·210	0·180	0·22	0·165
L-Rhamnose	0·59	0·59	0·37	0·285	0·220	0·30	0·180
D-Deoxyribose	0·73	0·60	—	—	—	0·32	—
L-Fucose	0·63	0·44	0·27	—	—	—	0·095
Lactose	0·38	0·24	0·09	0·0	0·0	0·070	0·0
Maltose	0·36	0·32	0·11	0·15	0·01	0·085	0·0
Sucrose	0·39	0·40	0·14	—	—	—	—
Raffinose	0·27	0·20	0·05	—	—	—	—

* All percentages are v/v.

greater the water content of the solvent phase. Since the amount of water carried by the solvent phase is dependent upon temperature, mobilities are higher the nearer the temperature of the chamber approaches the critical solution temperature of the solvent. Thus when phenol is used, an increase in temperature gives rise to increasing R_F values, while in *s*-collidine the effect is reversed. The temperature effect becomes less marked at working temperatures well below the critical point, and when phenol or collidine is used normal variations in the temperature of the room during a run can be tolerated. It is, however, advisable to lag the chamber in order to reduce such temperature disturbances to a minimum.

Since the differences in temperature between summer and winter working affect the R_F values of all the sugars to approximately the same degree, a standard sugar (glucose) was included in every chromatogram, and all R_F values obtained at temperature $t°$ were then multiplied by a factor a_t, where

$$a_t = \frac{R_F \text{ value of glucose at } 20°}{R_F \text{ value of glucose at } t°}.$$

Applying this correction it has been found that R_F values obtained on the same batch of filter paper are remarkably constant in spite of the uncontrollable variations in other factors met with in day-to-day working.

The behaviour of a solvent may be influenced by the addition to it of a small quantity of a solvent more readily soluble in water. This increases the solubility of water in the new solvent phase, and thus effects a general increase in R_F values. Thus the addition of acetic acid to *n*-butanol leads to an increase in R_F values and, in this case, to an improvement in the separations obtained with some of the sugars.

The behaviour of sugar mixtures

The R_F value given by a neutral sugar is not substantially affected by the presence of other sugars at comparable concentration in the same solution, although small deviations have sometimes been noted in the chromatograms given by solutions containing four or five different sugars. Pl. 2 (*a*) shows a 40 hr. chromatogram obtained in collidine with a solution containing ribose, mannose, fructose, galactose, lactose and galacturonic acid, the concentrations being 1% (w/v) with respect to each sugar. For the purpose of reference the individual sugars were run alongside the mixture. The photograph in Pl. 2 (*a*) should be compared with Fig. 1 (p. 242), which illustrates the degree of separation obtainable by use of two solvents, phenol-1% (w/v) NH_3 and collidine, and has been obtained by plotting the R_F values of the sugars in phenol as ordinates and the values in *s*-collidine as abscissae.

Sensitivity. Pl. 2 (*b*) shows a chromatogram obtained in collidine with a solution containing glucose (1% w/v) and xylose (1% w/v). The solution was applied in amounts varying from 4 μl. to 0·5 μl. The R_F values obtained by measuring the centre of the spots show no systematic variation with the amount of sugar present and this has been found to be the general rule. The amount of sugar applied (5–40 μg.) covers the range over which the method is useful for general purposes, but with some sugars (particularly glucose) much smaller amounts may be detected, and for special purposes considerably

larger amounts of sugar have been applied without causing serious distortion of the spots.

Interfering substances. Substances other than sugars present in the test solution may interfere in a variety of ways, and for the purpose of clarity they may be classified into groups as follows:

(*a*) Reducing substances which react with the ammoniacal silver nitrate reagent.

(*b*) Substances which may have mobilities close to those of the sugars it is desired to identify and which may affect the R_F values of the sugars by 'salting out'.

(*c*) Inorganic salts, which in addition to causing effects as under (*b*), may also be involved in reactions of the ion-exchange type leading to the localization of strong acids or bases which give rise to silver-reacting spots by modification of the cellulose of the filter paper.

Numerous substances occurring in tissue extracts were tested for their reaction with $AgNO_3$-ammonia under the conditions used in developing the chromatograms. Purines, creatine, creatinine and lactic acid and the common amino-acids were inactive, but inositol gave a weak brown spot developing at 110°, or at a rather lower temperature if a small concentration of NaOH was added to the spraying reagent. Ascorbic acid, gallic acid and tannic acid gave an immediate black coloration in the cold, and could readily be distinguished from the sugars by this means.

Interfering effects of class (*b*) were noticed in attempts to identify reducing sugars occurring in complex biological mixtures such as fruit juices or muscle press juice. In chromatograms obtained with the untreated extracts it was not uncommon to find that the spot due to a reducing sugar was badly distorted, or displaced by substances which revealed themselves as white spots or trails on the cream or buff-coloured ground of the chromatograms. As described below, reliable results could be obtained with such extracts only if steps were taken to remove the electrolytes, preferably by

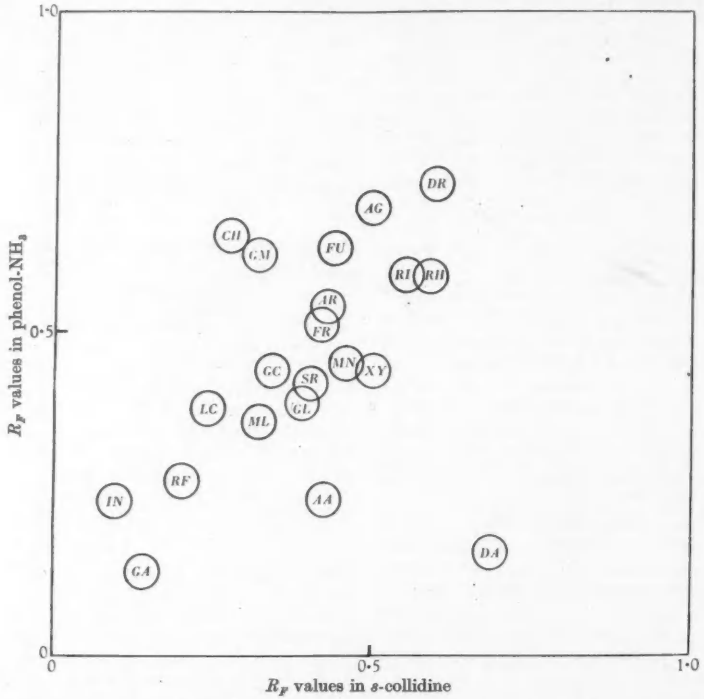


Fig. 1. Illustrating the separation obtainable by use of two solvents. The R_F values in phenol-1% NH_3 and *s*-collidine are plotted at right angles. *AA*, ascorbic acid; *AG*, acetylglucosamine; *AR*, arabinose; *CH*, chondrosamine; *DA*, dehydroascorbic acid; *DR*, deoxyribose; *FR*, fructose; *FU*, fucose; *GA*, galacturonic acid; *GC*, galactose; *GL*, glucose; *GM*, glucosamine; *IN*, inositol; *LC*, lactose; *ML*, maltose; *MN*, mannose; *RF*, raffinose; *RH*, rhamnose; *RI*, ribose; *SR*, sorbose; *XY*, xylose.

means of ion-exchange materials. However, the presence of such substances as the amino-acids could usually be tolerated provided their concentrations were not much greater than those of the sugars under test; this was particularly so if the non-sugar substances had R_F values either above or below the range occupied by the sugars.

The action of the paper chromatogram in separating the ions of inorganic salts is described in more detail by Westall in the note attached to this communication. When sufficient of the test solution was available the effect could be eliminated by making use of ion-exchange materials, and, in fact, the preliminary removal of all but small concentrations of electrolytes proved to be a necessary step before commencing the analysis of any unknown solution. With the hydrolysate of a pure carbohydrate, where the mineral acid may be removed quantitatively, the presence of interfering substances was not to be expected, and, in fact, in the example given in the second part of this communication (this vol. p. 251), the analysis of such a carbohydrate was uncomplicated by interference effects.

Reducing sugars with acidic or basic properties

The behaviour of the neutral reducing sugars on the filter-paper chromatograms was apparently uncomplicated by the various tautomeric equilibria known to occur in their aqueous solutions, and the spots revealed by the $AgNO_3$-ammonia reagent showed no tendency to trail. However, a few of the more common sugar derivatives with basic or acidic properties were investigated, and with some of these substances the products of reversible reactions taking place at or near room temperature showed some separation on the chromatograms. The results obtained with the sugar acids and amino sugars are given below; R_F values in the more useful solvents are shown in Table 2.

Glucuronic acid and its lactone. The source of glucuronic acid was L-menthol-D-glucuronide prepared biosynthetically by the method of Williams (1939). A sample of crystalline glucurone, $[\alpha]_D + 19{\cdot}7$, m.p. 178–180°, was prepared from this substance by hydrolysis with 0·2N-sulphuric acid according to the method of Quick (1927). The first hydrolysis product is a mixture containing glucuronic acid and its lactone in the proportion of 7 : 3, but crystallization from hot glacial acetic acid favours lactone formation and the product after two recrystallizations is the pure lactone.

The mixture of glucurone and glucuronic acid formed by first hydrolysis when applied to the filter-paper chromatogram gave rise to two well separated spots after development with the solvents (Pl. 3 (*a*) and (*b*)). The slower moving spot was due to glucuronic acid and was usually heart-shaped or elliptical, sometimes appearing with a marked forward trail. The spot due to glucurone had a high R_F value in all solvents and was invariably circular. The glucurone spot, however, developed more slowly than glucose after spraying with the silver reagent, and for this reason might easily be missed in the examination of the chromatograms.

Crystalline glucurone gave a single circular spot of high R_F in acidic solvents, but in basic solvents the spot was often accompanied by a backward trail, indicating conversion to glucuronic acid during the course of the run (Pl. 3 (*b*)). In the presence of ammonia vapour from a 1 % (w/v) aqueous ammonia solution, phenol behaves as a basic solvent, and the ammonia concentration is sufficient to complete the conversion of glucurone to the salt of glucuronic acid. Under these conditions the spot due to glucurone was entirely absent, the application of solutions of crystalline glucurone giving rise to the glucuronic acid spot accompanied by a forward trail.

Glucuronic acid reduced the $AgNO_3$-ammonia reagent very rapidly at 100°, but the lactone reduced only slowly at 110°. For this reason, when the lactone was suspected to be present, NaOH solution was added to the $AgNO_3$-ammonia reagent to bring the concentration of the NaOH to 0·05N. Glucurone was thereby converted on the filter paper to the sodium salt of glucuronic acid which rapidly reduced the silver reagent. Development of the colour was carried out at 110°, but the paper could not be heated longer than 3–5 min. because of excessive darkening. Butanol-acetic acid mixture was found the most suitable solvent for mixtures containing glucurone; the silver reagent containing sodium hydroxide was not suitable for use with phenol as the chromatographic solvent.

Galacturonic acid. D-Galacturonic acid does not readily form a lactone, and the crystalline acid gave rise to a single spot of low mobility which, like that due to glucuronic acid, was usually heart-shaped and often showed a short forward trail. Glucuronic acid and galacturonic acid may be differentiated by the formation of the glucurone spot by the former. R_F values are given in Table 2.

Glucosamine and chondrosamine. In basic solvents (phenol-NH_3 and collidine) the hexosamine hydrochlorides travelled as well defined circular spots which rapidly reduced the silver reagent at low temperatures (90°). In neutral or acidic solvents, however (phenol, butyric acid, butanol-acetic acid), the hexosamines appeared after development as brown spots with black forward trails. The trails reacted with the silver reagent at low temperatures and appeared much more rapidly than the spots during development. It appears, therefore, that the hexosamines travel as the free base under alkaline conditions, but in neutral or slightly acidic solvents give rise to equilibria between free base and cation which

result in partial separation, the free base travelling faster than the ion.

The Morgan & Elson reagents described above may be used to show the position of the spots due to the hexosamines, and in cases where the hexosamine spot is in close proximity to that due to another sugar, the specific reaction for the former is of considerable value.

N-Acetylglucosamine. This substance had a high mobility in most solvents, but gave a rather weak reaction with the ammoniacal silver nitrate reagent. However, the reaction with *p*-dimethylaminobenzaldehyde described above was very sensitive and also specific for the *N*-acetylhexosamines, and could be used with advantage wherever the *N*-acetyl derivatives were suspected.

Ascorbic acid. Freshly prepared aqueous solutions (1% w/v) of synthetic L-ascorbic acid were used. In phenol-ammonia, collidine and *n*-butanol-acetic acid, ascorbic acid gave well characterized spots which produced an immediate black colour on spraying with the $AgNO_3$-ammonia reagent in the cold. The presence of HCN was necessary when using phenol-ammonia as the chromatographic solvent in order to suppress catalytic oxidation of the ascorbic acid. The values for R_F found are given in Table 2.

Dehydroascorbic acid. Dehydroascorbic acid was prepared by oxidation of ascorbic acid with iodine according to the methods given by Herbert, Hirst, Percival, Reynolds & Smith (1933).

(*a*) An aqueous solution of ascorbic acid was oxidized with the calculated amount of iodine dissolved in ethanol, the addition being made at room temperature.

(*b*) An aqueous solution of ascorbic acid (10 ml. of 0·2M) was titrated with 0·2N-iodine in aqueous KI solution until the end point was reached. The solution had $[\alpha]_D + 53°$ (*l*, 2·0) (calculated on the weight of ascorbic acid taken) 30 min. after preparation. The solution was then titrated with N-NaOH to the temporary end point (phenolphthalein) in order to neutralize the mineral acid formed during the oxidation but to avoid salt formation with dehydroascorbic acid and consequent rupture of the lactone ring.

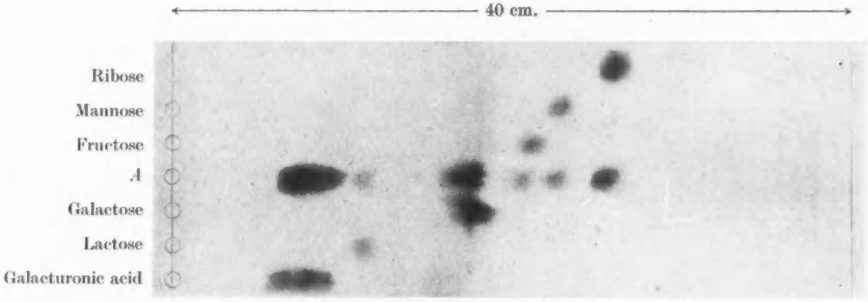
(*c*) A neutralized solution of dehydroascorbic lactone was prepared as in method (*b*), but the addition of N-NaOH was carried past the temporary end point until a permanent colour with phenolphthalein was obtained. The addition was carried out at room temperature. The solution contained the sodium salt of dehydroascorbic acid and was yellow in colour.

When freshly prepared the solutions (*a* or *b*) containing the lactone and that (*b*) containing the sodium salt of the free acid gave the same chromatogram, all three solutions yielding a single purple-brown spot which developed rapidly with the $AgNO_3$-ammonia reagent at 80–90°. This result was unexpected since glucuronic acid-glucurone mixtures gave rise to two distinct spots, widely separated in R_F value. However, inspection of Table 2 shows that the R_F values of dehydroascorbic acid in different solvents are markedly anomalous, since mobility is very high in collidine, but low in phenol-NH_3, and it is possible that the components of the system are too rapidly interconvertible to allow of the complete separation of either during the course of the run. In such a system the R_F value of the mixture would be expected to lie near that of the predominant component, and would therefore be markedly affected by any alteration in the solvent likely to disturb the equilibrium between the two components either in the solvent phase or in the water phase. The spots due to dehydroascorbic acid were usually

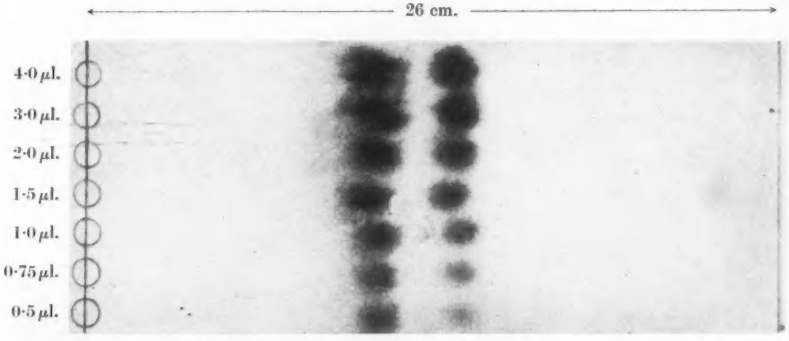
Table 2. *R_F values of a number of substances related to the sugars on Whatman no. 1 paper (corrected to 20°)*

Solvent	Phenol	*s*-Collidine	*n*-Butanol, 40%* Acetic acid, 10% Water, 50%	*iso*Butyric acid
Additions	1% NH_3, HCN	None	None	None
D-Galacturonic acid	0·13	0·14	0·14	0·09
D-Glucuronic acid	0·12	0·16 [0·72]†	0·12 [0·32]†	0·08 [0·22]†
D-Glucurone	0·12	— [0·72]†	— [0·33]†	— [0·22]†
D-Glucosamine hydrochloride	0·62	0·32	0·13 [0·17]‡	0·05 [0·20]‡
Chondrosamine hydrochloride	0·65	0·28	0·12 [0·16]‡	— [0·19]‡
N-Acetylglucosamine	0·69	0·50	0·26	0·25
L-Ascorbic acid	0·24	0·42	0·38	0·19
Dehydroascorbic acid	0·16	0·68	0·27	0·16
i-Inositol	0·23	0·10	0·09	—

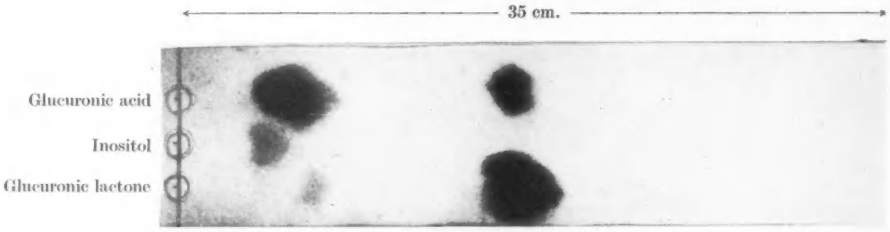
* All percentages are v/v.
† Values in brackets [ ] due to the lactone.
‡ Values in brackets [ ] due to free base; the two spots were connected by a trail.



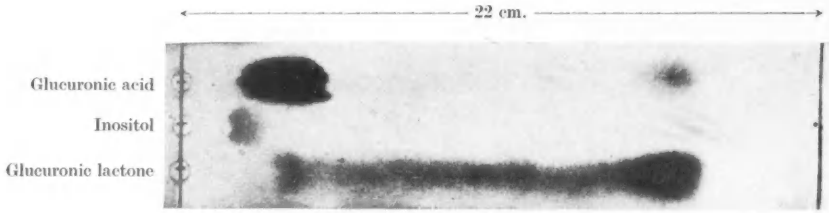
(*a*) Filter-paper chromatogram of a mixture containing six sugars. The mixture was introduced in the circle marked '*A*' and the individual sugars were placed in the other circles as indicated. The chromatogram was run 40 hr. in *s*-collidine.



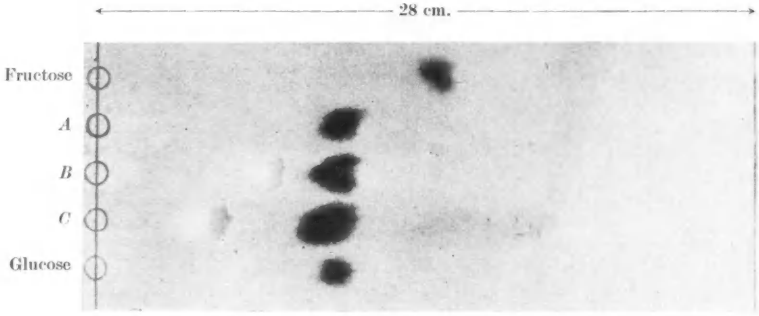
(*b*) Filter-paper chromatogram of a solution containing glucose (1% w/v) and xylose (1% w/v). The volume of solution applied to each circle is indicated. The chromatogram was run 20 hr. in *s*-collidine.



(*a*) Chromatogram run in butanol-acetic acid mixture. The chromatogram was sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.



(*b*) Chromatogram run in *s*-collidine and sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.



(*c*) Chromatogram of an extract of egg white in aqueous ethanol. *A*, extract treated with 'Zeo Karb 215' and 'Deacidite'. *B*, extract treated with 'Zeo Karb' only. *C*, untreated extract. ½The chromatogram was run 18 hr. in phenol-1% NH_3.

elliptical and showed marked forward trailing in collidine, but they were well separated from those due to ascorbic acid in most solvents.

Solutions (*a*) and (*c*) were unstable, and after keeping for 24 hr. gave traces of a second reducing substance yielding a slow-moving spot in collidine and butanol-acetic acid. This spot gave a brown colour with the silver reagent, but required heating to 100–110° for colour development. Solution (*a*) regenerated ascorbic acid on keeping for a few days at +2°, the solution giving rise to a third, small, but well defined spot which oxidized the silver reagent in the cold, and had an R_F value identical with that of ascorbic acid.

Inositol. The sample of *i*-inositol gave no reduction with alkaline ferricyanide (Hagedorn & Jensen, 1923) and was nitrogen-free. It was observed, however, that the substance gave a slow reduction with the $AgNO_3$-ammonia reagent under the ordinary conditions used in developing the paper chromatograms. The rate at which reduction took place was increased when NaOH was added to the $AgNO_3$-ammonia reagent to a concentration of 0·05 N, but even under these conditions the brown spot due to inositol was much less intense than that due to an equivalent concentration of a reducing sugar.

The mobility of inositol was low in all solvents (R_F values are given in Table 2), and consequently it was readily distinguished from aldoses and ketoses; but since the colour obtained with the silver reagent was not strong it was easily missed in the examination of chromatograms, particularly if present in the test solution in low concentration.

Application of method to extracts of biological origin

The object of this investigation was to establish a method for the qualitative analysis of polysaccharide hydrolysates. In such liquids the problem is relatively simple, since once the free mineral acid has been removed the solution is likely to be relatively free from salts, organic acids and other substances which might interfere. It was, however, considered of interest to explore the chromatographic analysis of a few examples of extracts of biological origin known to contain reducing sugars. The results of three of these investigations are given below.

Detection of glucose, fructose and sucrose in apple juice. An apple-juice extract was prepared from Bramley's Seedling apples and ethanol was added to a concentration of 80 % (v/v). After standing a few days at +2°, the solution was filtered, and the clear supernatant (500 ml.) retained for the analytical experiments. Standard methods of analysis showed: fructose, 0·34 %; glucose, 0·25 %; sucrose, 0·07 %; malic acid, 0·40 %; total dry solids, 2·45 %.

Part of the ethanolic solution was evaporated to small bulk under reduced pressure and made up with distilled water so that the resulting concentration was five times the original. This solution was clarified by centrifugation and used for the chromatographic experiments. In the description of the results which follows, the runs are classified according to the solvent used. This was usually neutral or alkaline phenol, since the best separation between glucose and fructose is obtained with phenol as the solvent.

(*a*) *Solvent, phenol; additions, none.* Brown spots of correct mobility for glucose and fructose could be distinguished readily, but they were much distorted in shape by the presence of other material of similar R_F value. Among the interfering substances observed were two well defined spots due to pigments of R_F 0·59 and 0·49, which gave transitory green or brown colours in the cold on spraying with $AgNO_3$-ammonia. In addition to the pigments there were a number of substances of low mobility which gave an immediate black colour in the cold on spraying with the $AgNO_3$-ammonia reagent. These were afterward shown to be tannins. There were further substances giving spots or trails which appeared colourless against the pale grey or cream colour of the paper background.

(*b*) *Solvent, phenol; additions,* NH_3 1 % (*w/v*), KCN. The appearance of the chromatograms was much improved in this solvent. Most of the acidic interfering substances had now very low mobilities due to the presence of the ammonia, and therefore did not interfere with the development of the spots due to the reducing sugars. Glucose and fructose gave well defined spots of correct R_F value, and the black colour due to the tannins was confined to a short trail springing from the position originally occupied by the test solution. However, since it was desired to test for the presence of other reducing sugars possibly present in small amounts, further runs were carried out with higher concentrations of the test solution. These were rather unsatisfactory, due to the simultaneous increase in concentration of interfering substances. For this reason an attempt was made to utilize ion-exchange reagents to remove acids, bases and salts while leaving the non-electrolytes in solution.

Use of ion-exchange columns. 'Zeo-Karb 215'* was used for removal of bases and cations and 'Deacidite'* for removal of acids. A portion (100 ml.) of the 80 % ethanolic apple-juice extract was evaporated to small bulk, and made up again to its original volume with distilled water in readiness to pass through the columns. In order to minimize the possibility of adsorption of sugars by the ion-exchange resins, sufficient of the test solution was

* Both these reagents may be obtained from Permutit Co. London.

passed to saturate the deacidite column with respect to anions, and the first runnings from both columns were rejected.

The columns were prepared by taking sufficient of the wet ion-exchange material to make up a volume of 22 ml. This gave a column 16 cm. in height when the material was packed into the filtration tubes. The deacidite was activated with 2N-NaOH solution, followed by thorough washing, while 2N-HCl solution was used to activate the zeo-karb. The test solution was passed slowly through the zeo-karb column and immediately transferred to the deacidite column. The pH of the solution flowing from the second column was followed by means of indicators, and the experiment was stopped when the reaction fell to pH 6·5, indicating approaching saturation of the deacidite. In all, 90 ml. of the solution were passed through both columns in this way, the first 15 ml. run from either column being rejected.

A portion (50 ml.) of the effluent was concentrated to 5 ml., and the small precipitate which formed was removed by centrifugation. The concentrated effluent solutions were tested by the filter-paper technique using phenol-NH_3, butanol-acetic acid mixture and collidine as solvents. The developed papers indicated freedom from interfering substances, and the R_F values given by glucose and fructose in the concentrates were not detectably different from those given by authentic glucose and fructose solutions run on the same chromatogram.

In order to ascertain if adsorption of reducing sugars by the columns was likely to be a serious consideration in further applications of the technique, the experiment was repeated under identical conditions except that 10 ml. of the test solution instead of 90 ml. were passed down the columns, and no part of the effluent was rejected. The relative intensity of the glucose and fructose spots was unaffected in the resulting chromatograms, and from this it was concluded that for qualitative purposes the method was satisfactory as far as glucose and fructose were concerned.

Detection of sucrose. Chromatograms were set up with the concentrated effluent from the ion-exchange columns used for the detection of glucose and fructose. Phenol and butanol-acetic acid were used as solvents and fructose, glucose and sucrose were included as reference sugars. After removal of the solvent by evaporation the chromatograms were sprayed with the naphthoresorcinol-trichloroacetic acid reagent as described in the experimental section. The spots due to fructose and sucrose in the mixture gave strong red colorations and had R_F values identical with those of the reference sugars. Glucose gave no colour with this reagent.

Detection of glucose in the white of egg. The white was separated from a hen's egg taken a few hours after laying. After dilution with water to 100 ml., absolute ethanol (400 ml.) was added with stirring and the precipitate allowed to settle. The coagulated proteins were filtered off and washed with a small volume of 80 % (v/v) aqueous ethanol. The ethanolic extract was then allowed to stand for a few days at +2°, when it was refiltered and evaporated to 60 ml. under reduced pressure. Part of the concentrated solution (20 ml.) was further evaporated under reduced pressure to 2 ml. (solution (*a*)). A second part (40 ml.) was passed through a column packed with activated 'Zeo-Karb 215' (wet volume 9 ml.) and the first 5 ml. of the effluent rejected. The effluent solution was then divided into two equal parts and one portion concentrated to 2 ml. (solution (*b*)). The remainder of the solution was passed through a column packed with prepared 'Deacidite' (wet volume 10 ml.), the first 6 ml. of the effluent being rejected and the remainder concentrated to 2 ml. (solution (*c*)). Passage through the zeo-karb column removed a yellow pigment from the solution and CO_2 was evolved; the effluent from the deacidite column was clear and colourless.

Chromatograms were prepared using phenol-NH_3 and butanol-acetic acid mixture as solvents, the three solutions being run side by side on the same paper strip. All three solutions showed well characterized spots corresponding to glucose (Pl. 3 (*c*)); fructose was not found. Grey-brown irregular spots due to interfering substances were visible with (*a*) and with (*b*) but were absent from (*c*).

Detection of fructose, glucose and inositol in the foetal blood of the sheep. The presence of fructose as well as glucose in the foetal blood of the sheep was demonstrated by Bacon & Bell (1946) who isolated the 2 : 3, 4 : 5 diacetone derivative of fructose (Bell, 1947). A laevorotatory sugar had previously been reported by Paton, Watson & Kerr (1907). An extract of whole foetal blood that had been deproteinized by ethanol-chloroform treatment was kindly supplied by Dr J. S. D. Bacon. This was prepared from 50 ml. of blood and contained, in addition, water (150 ml.), ethanol (100 ml.) and chloroform (50 ml.). The solvents were removed by evaporation and the solution made up to 40 ml. A portion of the aqueous solution (20 ml.) was passed through a column containing activated zeo-karb (3 g.) and then through deacidite (5 g.), following the procedure described above for white of egg. The salt-free effluent from the columns was concentrated under reduced pressure to a volume of 2–3 ml., after which it was evaporated to dryness under vacuum at room temperature and the dry residue made up to 0·25 ml. with distilled water.

Chromatograms in three solvents, phenol, collidine and butanol-acetic acid, showed the presence of glucose, fructose and inositol when sprayed with the ammoniacal silver nitrate reagent. Since the

spot due to inositol was light in colour, further chromatograms were sprayed with ammoniacal silver nitrate containing NaOH to a concentration of 0·05N, and in these the inositol showed strongly in the mixture and had R_F values identical with the values given by authentic specimens. Further chromatograms run in phenol and butanol-acetic acid were sprayed with naphthoresorcinol-trichloroacetic acid mixture. These showed the presence of fructose as red spots of R_F value identical with those due to authentic specimens of the sugar.

The procedure given above was repeated with samples of blood from three normal adult sheep (not pregnant), and in each case glucose only was found; fructose and inositol appeared to be absent or in quantities too small to be detected.

DISCUSSION

In Fig. 1 the R_F values of the reducing sugars in phenol-NH_3 have been plotted as ordinates and the values in *s*-collidine as abscissae. The circles drawn around each point give an approximate indication of the relative size of the coloured spot normally encountered, so that the figure gives a general indication of the degree of separation obtainable with the two solvents.

With two solvents only, complete separation between several pairs of sugars is not obtainable, but other solvents and solvent mixtures allow of further separation. A useful third solvent is *n*-butanol-acetic acid mixture, and experience has shown that it is usually necessary to run chromatograms with at least three solvents if unambiguous identifications are to be ensured.

With none of the solvents tested has complete separation been obtained on 18–20 hr. chromatograms between sorbose and glucose, arabinose and fructose or chondrosamine and glucosamine, so that clear identification is not yet possible when both sugars of any of these pairs appear in a solution together. However, when only one sugar of such a pair is present the separation is usually sufficient for successful identification by reference to the R_F values in several solvents.

Some inference as to the effect of chemical constitution on R_F values may be drawn from the spread of the sugar spots as displayed in Fig. 1. In general, as would be expected, the more strongly hydrophilic substances are the more slowly transported. The carboxylic acid group is particularly effective in giving rise to a low R_F value as indicated by the slow movement in both solvents of the uronic acids. When an —OH group is replaced by —NH_2 as in the hexosamines, a higher R_F value results with phenol but not with collidine. Still higher values with both solvents may be obtained by replacement of —OH by H as in the deoxysugars.

The aldohexoses occupy roughly the centre of the scale, but overlap with the ketoses. The pentoses appear to be rather faster and the disaccharides maltose and lactose are slower. However, it is characteristic of the results that the sugars most difficult to separate are not necessarily members of the same group in the usual chemical classification. The behaviour of dehydroascorbic acid appears to be anomalous, since it is slow in phenol, but has a very high mobility in collidine. This may be due to the intervention of the lactone structure.

The bearing of various external conditions upon the reproducibility of the R_F values of the amino-acids has been discussed by Consden *et al.* (1944), and since the same considerations apply with equal force to the behaviour of reducing sugars the subject need not be considered in detail here. It is sufficient to point out that variations in R_F value due to changes in temperature and other factors may be considerable, and in the analyses described in the experimental section identification of the components of a mixture was always based on the R_F values of authentic specimens of the sugars when run side by side on the same filter-paper strip. In order to compensate for temperature variations, a standard sugar (glucose) was put up on every chromatogram, and all R_F values were corrected to 20° by reference to the standard sugar.

It is apparent from inspection of Fig. 1 that where more than a small number of reducing sugars appear in the same solution there would be an advantage in carrying out two-dimensional chromatograms, following the technique described by Consden *et al.* (1944). A few such chromatograms have been prepared, the spot containing the mixed sugars being developed in one direction with phenol and afterwards in a direction at right angles with collidine. The neutral reducing sugars gave encouraging results, but it was found that in the case of amino sugars too much spreading of the spot took place, with the result that the sugars could not be identified accurately by reference to their R_F values. The two-dimensional technique appears to be less reliable than the filter-paper strip method where the test solution contains a small concentration of one component, and since more than two or three different sugars are rarely found in natural mixtures, it has generally been considered preferable to apply the filter-paper strip method rather than the more time-consuming two-dimensional technique.

The R_F values given by the sugars are little affected by the presence of other sugars even though they may appear in the test solution in much larger quantities; in one experiment, glucose showed its normal R_F value in the presence of a concentration of lactose 50 times greater. Interference effects by electrolytes however are serious, and in carrying out the analysis of solutions of biological origin con-

taining sugars it is almost always necessary to effect the prior removal of salts, acids and bases. A convenient way in which this may be done is by making use of ion-exchange reagents. For this purpose two synthetic ion-exchange resins were found to be suitable. Cations may be removed from neutral solution by passage through a column of the Permutit product 'Zeo-Karb 215' (Permutit Co., London), while the anions may be removed from the resulting acidic solution by similar treatment with 'Deacidite'.

The neutral reducing sugars give rise to essentially circular spots with sharp edges under the conditions described, and although various tautomeric reactions are possible with these substances, such reactions do not appear to affect the transport of the sugar as a single component. This is not altogether the case with the amino sugars or the uronic acids. The hydrochlorides of glucosamine and chondrosamine form short trails with acidic or neutral solvents but give well formed spots under conditions of sufficient alkalinity to suppress the cation. The uronic acids show a tendency to form heart-shaped spots with basic solvents, and in the case of glucuronic acid behaviour is complicated by the presence of a slow reversible reaction between free glucuronic acid and its lactone. In acidic solvents solutions of glucuronic acid give rise to two well separated spots, one of them due to the lactone. Galacturonic acid, on the other hand, does not readily form a lactone, and for this reason it can be differentiated from glucuronic acid in spite of the fact that the R_F values of the free acids lie close together.

A number of compounds not strictly belonging to the sugar series were briefly investigated, since they may be met with in the analysis of extracts of biological origin. Three such compounds were ascorbic acid, dehydroascorbic acid and inositol; however, it was found that although all three substances travelled as fairly well defined spots, the R_F values were well outside the range occupied by the reducing sugars. Ascorbic acid showed a very characteristic reaction with the ammoniacal silver nitrate reagent, giving an immediate black coloration in the cold, while, on the other hand, inositol reduced the silver reagent very slowly at 110°, and could only be detected when present in rather high concentration. As examples of the application of the technique, a number of extracts of biological origin have been examined, and three such analyses are reported in detail in the experimental section. Glucose, fructose and sucrose were identified in an 80 % (v/v) ethanolic extract of Bramley's Seedling apples, while an examination of the soluble sugars of fresh white of egg showed the presence of glucose only, no fructose being detected. In an ethanol-chloroform extract of whole blood from the foetus of a sheep the presence of fructose and glucose was confirmed, and in addition inositol was found. In all three cases it was necessary to remove interfering electrolytes by treatment with ion-exchange reagents.

The second part of this paper (this vol. p. 251) describes two examples of the application of the method to the analysis of the hydrolysis products of polysaccharides.

SUMMARY

1. A method is described for the identification of reducing sugars in mixtures in which use is made of the filter-paper chromatogram introduced by Consden *et al.* (1944).

2. The use as a spraying reagent of a solution of silver nitrate in ammonia was found to be effective as a general method for revealing the position of the sugars on the chromatogram, and other spraying reagents useful in special circumstances are described.

3. The behaviour on the filter-paper chromatogram of twenty-two sugars and sugar derivatives has been investigated using several solvents, and the degree of separation obtained has been indicated.

4. The effect of interfering electrolytes is discussed, and a method of removing them by application of ion-exchange reagents is described.

5. As examples of the technique, the analysis of three extracts of biological origin is described; glucose only was found in an 80 % (v/v) ethanolic extract of fresh white of egg, while glucose, fructose and sucrose were shown in a fraction from Bramley's Seedling apples. The presence of glucose, fructose and inositol was shown in an extract from the foetal blood of the sheep.

The author wishes to express his thanks to Dr D. J. Bell and Dr J. S. D. Bacon for their interest in the work, and for the gift of some of the specimens of pure sugars used in this investigation.

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Correction added 12 March 1948

The R_F values for dehydroascorbic acid given in Table 2 above were noted to be anomalous and reinvestigation has revealed an error due to the interference of I′ from NaI in the solution. Although NaI gives no brown colour when sprayed with $AgNO_3$-ammonia under the conditions used, it has now been shown that on irrigation with the chromatographic solvents the ions of the salt migrate at different speeds, each forming a discrete spot on the paper. On spraying with $AgNO_3$-ammonia the reagent reacts with HI derived from I′ to give an intense purple-brown colour. Cl′ is inactive, but Br′ gives a blue-grey colour when present in high concentration.

The R_F values for dehydroascorbic acid should be corrected as follows: collidine, 0·47; phenol (additions: NH_3 (1%), HCN) 0·28; butanol-acetic acid, 0·48.

Addendum. Note on the Behaviour of Inorganic Salts on the Filter-paper Partition Chromatogram

By R. G. WESTALL, *Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge*

During the course of some work on the dialyzable fraction of fresh rabbit muscle, the chromatographic method described in this paper was applied to the identification of the reducing substances. Application of the routine procedures detailed above led to the expected identification of glucose, but in addition an unknown substance was present which gave rise to a heavy spot on the chromatogram after spraying with $AgNO_3$-ammonia. The substance had an R_F value of 0·59 in phenol-1 % NH_3, 0·09 in *s*-collidine and 0·20 in butanol-acetic acid mixture and was adsorbed by the cation exchange reagent 'Zeo-Karb 215'. It was therefore considered to be basic in character.

The behaviour on the filter-paper chromatogram of a number of bases known or suspected to be present in muscle juice was then investigated. The R_F values and the spraying reagents used to reveal the positions of the spots are given in Table 1. The strong bases spermine and spermidine gave brown spots when treated with $AgNO_3$-ammonia, but it was also found that strong inorganic bases, which would not be expected to reduce silver nitrate, gave similar brown colorations. In order to demonstrate this effect, various dilutions of NaOH and KOH were introduced in spots as duplicates on two strips of filter paper. One strip was heated at 105° for 1 hr. (the normal time allowed to remove the solvent from a filter-paper chromatogram), while the other was not heat treated. Both strips were sprayed with $AgNO_3$-ammonia and placed in the oven for 10 min. to develop the colour. The spots on the heat-treated paper reacted strongly to the silver reagent, whilst those on the control strip reacted only slightly. From this it appeared that the effect of the alkali was to modify the cellulose of the paper during the heat treatment required to remove the solvent from the chromatogram. This resulted in the liberation of reducing substances that were responsible for the reduction of the silver reagent.

Table 1. *R_F values of some substances present in muscle*

Substance	Phenol-NH_3	*s*-Collidine	Butanol-acetic acid	Reaction with $AgNO_3$-NH_3	Reagent used for spraying	Colour of spot
Anserine	0·92	0·10	0·18	0	Ninhydrin	Red-brown
Carnosine	0·83	0·09	0·20	0	Ninhydrin	Red-brown
Creatine	0·82	—	—	0	Picric acid reagent	Orange
Creatinine	0·94	—	—	0	Picric acid reagent	Orange
Urea	0·73	0·44	0·43	0	Ehrlich's reagent	Grey
Spermine	0·95	0·01	0·03	+	Bromothymol blue	Blue
Spermidine	0·95	0·03	0·07	+	Bromothymol blue	Blue
Glucose	0·39	0·39	0·18	+	$AgNO_3$-NH_3	Brown
NaCl-KCl	0·59	0·09	0·20	+	Bromothymol blue	Blue

Neutral inorganic salts, when introduced on to a test sheet of filter paper, gave no colour with $AgNO_3$-ammonia after similar heat treatment, but if applied to a chromatogram and irrigated with phenol-1 % NH_3 they gave rise to heavy black spots after removal of the solvent and spraying with the reagent. This effect appeared to be due to hydrolysis of the salt with separation of the resulting strong acid and strong base. It was found that the area containing free base could be demonstrated by application of a number of common indicator dyes, a solution of bromothymol blue in a mixture of butanol and ethanol being particularly useful as a spraying agent. Solutions (0·01 M) of NaCl, KCl, $MgCl_2$, $CaCl_2$ and $BaCl_2$ were applied as spots to two filter-paper strips, and both strips were irrigated with phenol-1 % NH_3 (18 hr.). After removal of the solvent at 105°, one strip was sprayed with bromothymol blue solution and the other with $AgNO_3$-ammonia. The paper sprayed with the indicator showed a series of dark blue spots on a green ground, while that sprayed with the silver reagent gave a corresponding series of brown spots. The spots had short backward trails, but the leading edges were sharp. There was little differentiation of R_F values, all the salts giving rise to alkaline areas at R_F 0·55–0·58. The amount of salt applied to the chromatogram was very small, being in each case not more than 2–4 μg.

Not all indicator dyes were suitable for use in showing the presence of acids and bases on the chromatograms, since the behaviour of the dyes appeared to be affected by the cellulose itself, and by the presence on it of residual traces of the solvent used in developing the chromatogram. Suitable dyes were found by trial and error. Bromothymol blue

was satisfactory for strong bases both with phenol-1% NH_3 and with collidine as chromatographic solvents; in the case of collidine chromatograms the alkaline spots showed blue on a yellow ground.

The identity of the unknown spot on the chromatograms of muscle juice and the spots given by chlorides of sodium and potassium was shown by a comparison of the chromatograms in three different solvents, phenol-1% NH_3, collidine and butanol-acetic acid mixture. When the solutions were developed side by side on the same paper strips, identical R_F values were obtained. The spot given by the inorganic salts in muscle juice was sufficiently intense to react strongly with the bromothymol blue indicator, and this reagent has since been used to confirm the basic character of other spots suspected as being due to interference by inorganic salts. However, for the purpose of identifying the reducing sugars in a mixture, it is more satisfactory to ensure the removal of all but traces of inorganic salts by preliminary treatment with suitable ion-exchange reagents.

The process of separation of the ions of a salt on the paper chromatogram may be regarded as analogous with that occurring on ion-exchange reagents, and is of particular interest since both products of hydrolysis of the salt appear as narrow moving bands.

A simple explanation of the phenomenon lies in a consideration of the equilibrium

$$NaCl + C_6H_5OH \rightleftharpoons C_6H_5ONa + HCl.$$

Here, undissociated sodium phenate would be expected to have a high R_F value in phenol, and the equilibrium would be moved to the right as a result of transport of sodium phenate out of the system. A mechanism of this sort is rendered plausible, since in phenol-NH_3 and butanol-acetic acid the sodium ion travels farther than the chloride ion, while in the basic solvent collidine the reverse is the case. Table 2 shows the separation of sodium and chloride ions obtained with a number of solvents, and it will be observed that in phenol-NH_3 the R_F values of Na^+ and Cl^- from sodium chloride are identical with the values given by Na^+ and Cl^- from sodium hydroxide and hydrochloric acid. In butanol-acetic acid and *s*-collidine, separation is not so complete, and the spots due to Na^+ and Cl^- from NaCl occupy intermediate positions between those due to the same ions from NaOH and HCl. The presence of ammonia vapour has a marked effect on the separation taking place with phenol as solvent, since in its absence separation is very poor, and with sodium chloride only light trails due to NaOH and HCl are detectable.

Table 2. *R_F values given by* Na^+ *and* Cl^- *with various solvents*

	Phenol-NH_3		*s*-Collidine		Butanol-acetic acid	
	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)
NaOH	0·61	—	0·07	—	0·23	—
NaCl	0·61	0·17	0·15	0·38	0·20	0·15
HCl	—	0·17	—	0·43	—	0·25

The possibility of the intervention of cellulose itself, acting as an ion-exchange reagent, should be taken into account in discussing the mechanism of the separation, and the distribution of the ions between the phases will also be affected by the potential existing at the surface of shear between the wet cellulose and the moving solvent. It is probable, therefore, that the explanation suggested above presents a considerable over simplification of the problem.

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Filter-paper Partition Chromatography of Sugars

2. AN EXAMINATION OF THE BLOOD GROUP A SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN AND THE SPECIFIC POLYSACCHARIDE OF *BACTERIUM DYSENTERIAE* (SHIGA)

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The primary object of this investigation was to assess the value of the chromatographic technique described in the preceding paper (this vol. p. 238) as a qualitative method for the analysis of the hydrolysis products of complex polysaccharides. Interest especially centres in the general class of mucopolysaccharides—polysaccharides which contain hexosamines and which frequently show a special capacity for forming combinations with proteins—the reactions of which may be of considerable importance in the storage behaviour of foodstuffs. The specific polysacharide prepared from the 'O' somatic antigen of *Bacterium dysenteriae* (Shiga) and the blood group 'A substance' from hog gastric mucin were chosen as being polysaccharides of this class which were available in a condition of presumed homogeneity and which had already been investigated by the classical methods.

The occurrence of complex polysaccharides having blood group A activity in certain mucinous secretions of man and animals has been dealt with in recent reviews by Wiener (1943) and Morgan (1945). A chemical investigation of the glycoproteins of hog gastric mucin was undertaken by Meyer, Smyth & Palmer (1937), who found the neutral carbohydrate portion of their product to consist of equimolecular proportions of galactose and *N*-acetylglucosamine. Later, Landsteiner & Hart (1940) showed that the material had blood group A activity and contained a component rich in amino-acids.

Morgan & King (1943) and Morgan (1944) extended these observations by preparing a highly purified and essentially undegraded specific substance from hog gastric mucin which contained some 15 amino-acids in addition to the sugar residues.

The constitution of the carbohydrate portion of the complex was further studied by Bray, Henry & Stacey (1946 *a*, *b*) who isolated material showing blood group A activity from a number of different sources. The group A specific substance from pepsin was studied by the method of methylation and hydrolysis, with subsequent isolation of the methylated sugars. Four sugar constituents were found, viz. glucosamine, galactose, fucose and mannose. A parallel experiment was carried out with the blood group 'A substance' isolated by Morgan & King from hog gastric mucin, and from this substance also a fully methylated L-fucose derivative was obtained in 6% yield, but the remaining sugar constituents are not stated.

It has since been pointed out (Aminoff, Morgan & Watkins, 1946) that the apparently homogeneous specimens of 'A substance' isolated from commercial hog gastric mucin possess both A and O blood group specificity. Mucoids with A or O specificity only may be prepared from the stomachs of individual hogs and it is probable that commercial hog gastric mucin, which is derived from many animals, contains a mixture of two mucoids of very similar chemical and physical properties but of differing immunological specificity.

The sample of bacterial specific polysaccharide used in the present investigations was supplied by Dr W. T. J. Morgan. It was obtained from the somatic 'O' antigen of *Bact. dysenteriae* (Shiga) (Morgan, 1936, 1937) by gentle hydrolysis with acetic acid, and has been shown to contain L-rhamnose, D-galactose and an acetylhexosamine believed to be *N*-acetylglucosamine (Morgan, 1938).

EXPERIMENTAL

Blood group A substance

The sample of blood group A substance was kindly supplied by Dr W. T. J. Morgan. It had been obtained from commercial hog gastric mucin by extraction with phenol and was shown to be electrophoretically homogeneous at pH 4 and 8. On hydrolysis with 0·5 N-HCl at 100° the reducing sugar reached its maximum after 4 hr., when the reducing value (expressed as glucose) was 50%.

The substance (30 mg.) was sealed with 1·5 ml. of 0·5 N-H_2SO_4 in an ampoule and heated for 4 hr. at 100°. 1 ml. of the hydrolysate was brought to pH 4·5 with $Ba(OH)_2$ solution and the $BaSO_4$ removed by centrifugation. The clear solution, together with the washings, was concentrated to dryness under vacuum at room temperature and made up to 0·5 ml. in readiness for application to the chromatograms. Phenol-1% (w/v) NH_3, *s*-collidine and butanol-acetic acid mixture were used as solvents for the

chromatograms and glucose, D-glucosamine hydrochloride, chondrosamine hydrochloride, L-fucose, D-galactose and D-mannose were included as reference sugars.

s-Collidine. The chromatograms gave four spots corresponding to fucose, galactose, glucosamine and an unknown reducing substance giving a well defined circular spot at R_F 0·26. Mannose was absent.

Phenol-1% (*w/v*) NH_3. The chromatogram showed three well defined spots corresponding to fucose, glucosamine and galactose. Chondrosamine was absent. Mannose was not detectable since it would be obscured by galactose.

Butanol-acetic acid. The chromatogram gave clear indication of fucose, galactose and glucosamine. Mannose was absent, but an unknown reducing substance yielded a spot at $R_F = 0{\cdot}055$.

The principal sugars present were galactose and glucosamine, fucose appeared to be present in smaller amount. In case hydrolysis had not been completed under the conditions used for the above series, a further sample of the polysaccharide was hydrolyzed for 10 hr. with N-H_2SO_4, and the hydrolysate treated as before. The resulting chromatograms were similar to those previously obtained except that the spot given by fucose was relatively lighter, and that given by glucosamine was heavier.

The R_F value of the unknown reducing substance was below the range normally encountered for hexoses, pentoses and amino sugars, and the presence of a reducing disaccharide was considered unlikely since the unknown spot persisted after hydrolysis under more vigorous conditions.

Treatment with cation-exchange resin. The hydrolysate from 30 mg. of the polysaccharide (contained in 3 ml. solution) was shaken for 30 min. with an activated and washed sample of 'Zeo Karb 215' (0·5 g.) as described in the previous communication. The concentrated filtrate was then applied to further chromatograms. The treatment with ion-exchange resin resulted in removal of the unknown material together with glucosamine, and the chromatograms in all three solvents showed two spots only, corresponding to galactose and fucose (Pl. 4 *a*). It is probable, therefore, that the unknown substance is basic in character.

The experiments described above indicate clearly the presence of galactose, glucosamine and fucose, but give no indication of the presence of mannose in the sample of blood group A substance used for the investigation.

The specific polysaccharide of Bact. dysenteriae (*Shiga*)

The polysaccharide had N 1·6% and yielded 97% of reducing substances calculated as glucose after acid hydrolysis (Hagedorn & Jensen). A solution of the material (10 mg.) in 0·5 N-H_2SO_4 (2 ml.) was sealed in an ampoule and heated at 100° for 5 hr. The hydrolysis mixture was clear and colourless and a measured volume (1 ml.) was neutralized to pH 4·5 with $Ba(OH)_2$ solution. After removal of the $BaSO_4$ the solution, together with the washings, was transferred to a small crucible and evaporated to dryness under vacuum at room temperature. The dry residue was dissolved in water (0·1 ml.) and the solution stored at +2° over chloroform. Where one-dimensional chromatograms were applied, the following sugars were included as reference substances; glucose, D-glucosamine hydrochloride, *N*-acetylglucosamine, chrondrosamine hydrochloride, L-rhamnose and D-galactose. Collidine, phenol-1% (w/v) NH_3 and butanol-acetic acid mixture were used as solvents.

s-Collidine. The developed chromatogram showed two spots. One of them at R_F 0·59 corresponded to rhamnose, while the second spot at R_F 0·32–0·34 appeared to be multiple. The slower moving substance gave rise to a black coloration with $AgNO_3$-ammonia as soon as the temperature of the oven reached 90° and later (at 105°) the spot extended in size with development of a brown colour.

It was considered that the multiple spot at R_F 0·32–0·34 was probably due to both glucosamine and galactose, and for this reason the experiment was repeated using a spraying reagent, based on the Morgan & Elson colour reaction for hexosamines. (Details are given on p. 240 of this volume.) In order to show the presence of *N*-acetylhexosamines, a chromatogram was sprayed with the modified Ehrlich's reagent alone, while to show both free hexosamine and the *N*-acetylhexosamines a second chromatogram was sprayed with the alkaline acetylacetone reagent, followed by the acid Ehrlich's reagent. Under these conditions the spot at R_F 0·33 showed the presence of free glucosamine in small quantity while a third spot appeared at R_F 0·50 corresponding to *N*-acetylglucosamine.

Phenol-1% (*w/v*) NH_3. With this solvent galactose was shown unambiguously, but the rhamnose spot was confused with that due to glucosamine (Pl. 4 *b*). As before both free glucosamine and *N*-acetylglucosamine could be detected as red-violet spots by means of the modified Morgan & Elson reagents.

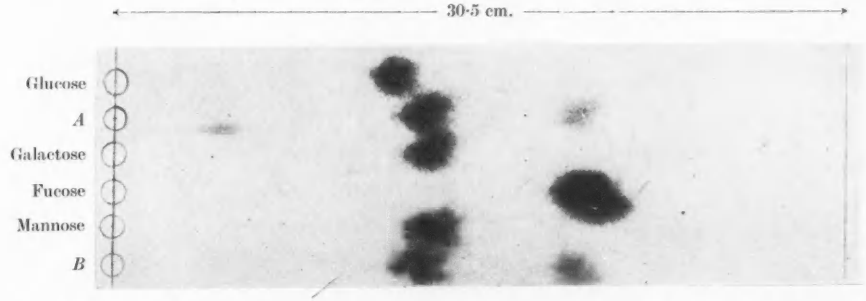
Butanol-acetic acid. Like collidine this solvent mixture led to the unambiguous identification of rhamnose, but as in the case of collidine, the spot due to glucosamine was confused with that due to galactose.

Since *N*-acetylglucosamine was found it was evident that the conditions of hydrolysis were not sufficiently severe to complete the deacetylation of the amino sugar. For this reason the experiments described above were repeated, hydrolysis being carried out with N-H_2SO_4 for 10 hr. at 100°. The hydrolysate no longer showed the presence of *N*-acetylglucosamine, and the spots due to free glucosamine were correspondingly heavier. Galactose and rhamnose appeared in much the same concentration as before.

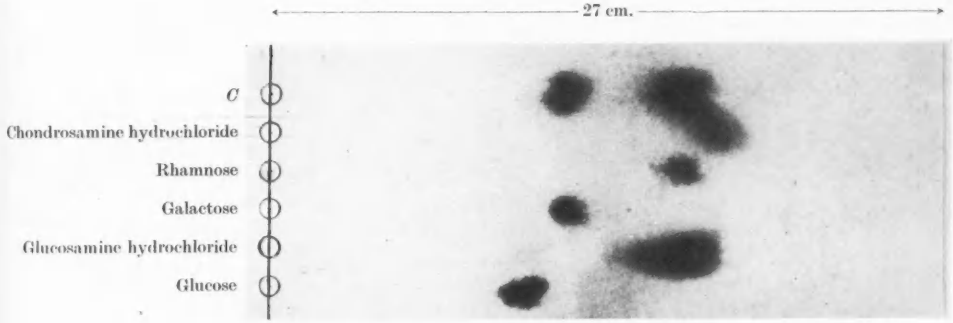
As confirmatory evidence, a two dimensional chromatogram was set up with a hydrolysis mixture prepared by heating the polysaccharide with N-H_2SO_4. The chromatogram was run first for 24 hr. with phenol-1% (w/v) NH_3, and after drying turned through 90° and irrigated with collidine for a further period of 24 hr. The chromatogram showed two heavy spots, which could be identified from their co-ordinates as galactose and rhamnose. The glucosamine appeared as a diffuse trail which was unsuitable for the purpose of identification from co-ordinates.

DISCUSSION

As would be expected in the case of a homogeneous polysaccharide, the identification of the sugars present in the product of hydrolysis of the specific substance from *Bact. dysenteriae* (Shiga) was uncomplicated by the presence of interfering substances; galactose, glucosamine and rhamnose could readily be recognized by reference to the respective R_F values in each of three solvents. The presence of the hexosamine was confirmed by spraying the developed chromatograms with alkaline acetyl-



(*a*) Hydrolysate of blood group A specific substance after treatment with ion-exchange resins; showing the presence of galactose and fucose. The hydrolysate after treatment with 'Zeo-Karb 215' was placed in the circle marked '*A*'. *B* shows position of a hydrolysate treated with both 'Zeo-Karb' and 'Deacidite'. Chromatogram run with phenol for 20 hr.



(*b*) Hydrolysate of polysaccharide from *Bact. dysenteriae* (Shiga); showing presence of galactose and a multiple spot due to rhamnose and glucosamine. The hydrolysate was placed in position marked '*C*'. Chromatogram run with phenol for 16 hr.

acetone followed by an acid solution containing *p*-dimethylaminobenzaldehyde, and as further confirmation the presence of galactose and rhamnose was shown by carrying out a two-dimensional chromatogram according to the technique described for amino-acids by Consden, Gordon & Martin (1944).

With the blood group A substance, the analysis was complicated by the fact that only some 70% of the material was carbohydrate in nature, and an interfering substance was found which gave rise to a spot at R_F 0·26 in collidine after spraying with the ammoniacal silver nitrate reagent. This substance was removed by treatment with a cation-exchange resin, and was therefore considered to be basic in nature. The analysis showed clearly the presence of glucosamine, galactose and fucose, but, in spite of indications in the literature of the presence of mannose in allied blood group A substances, this sugar was not found in the hydrolysis products of the polysaccharide under investigation.

The analysis of these two trial substances indicates clearly the importance of control of the hydrolysis conditions, and in assessing the results of such analyses it should be borne in mind that the hydrolysis of complex polysaccharides may proceed in stages, some destruction of the less stable sugar residues taking place before complete liberation of residues constituting a resistant core.

SUMMARY

1. The method of separating reducing sugars on filter-paper partition chromatograms described in this volume p. 238 has been applied to the analysis of the products of hydrolysis of two carbohydrate-containing materials: (*a*) the specific polysaccharide of *Bact. dysenteriae* (Shiga), and (*b*) the blood group A substance from hog gastric mucin.

2. The results of the analysis of (*a*) agree with results in the literature in showing the presence of glucosamine, galactose and rhamnose.

3. In the hydrolysis products of (*b*) glucosamine, galactose and fucose were found. Mannose, which has been observed in active polysaccharide fractions from other sources, was not found in the specific substance from hog gastric mucin.

The author wishes to express his thanks to Dr W. T. J. Morgan for his interest and for the gift of the polysaccharides used in this investigation.

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Urinary Elimination of Synthetic Oestrogens and Stilboestrol Glucuronide in Animals

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Clinical experience has shown that oestrogen treatment of carcinoma of the prostate fails after a varying lapse of time in many cases. Although a similar delayed recurrence of symptoms also frequently follows treatment of prostatic carcinoma by castration (Angrist & Khoury, 1944; Nesbit, Pazzos & Cummings, 1944; Huggins, 1946) there is a slight possibility that the failure of continued oestrogen treatment (in spite of increased dosage) is due to some bodily adaptation involving an altered metabolism of the drug. Hence it was thought worth while to study the metabolism of synthetic oestrogens in patients and animals; at the same time the opportunity has been taken to collect the monoglucuronides of dienoestrol (4:4′-dihydroxy-γ:δ-diphenylhexa-β:δ-diene), hexoestrol (4:4′-dihydroxy-

γ:δ-diphenyl-*n*-hexane), and stilboestrol (4:4′-dihydroxy-α:β-diethylstilbene) for chemical and biological investigation (Simpson & Wilder Smith, 1948).

The metabolism of the natural oestrogens has been widely studied, but that of the synthetic oestrogens comparatively little. Stroud (1939) found that in the rabbit 21·3% of a high dose of hexoestrol was excreted in the urine, 15·7% in the free state and 5·6% in a conjugated form. The corresponding figures for stilboestrol were 14·2 and 4·2%, and for dienoestrol 11·0 and 3·0%. In view of the instability of dienoestrol (Wilder Smith & Williams, 1945; 1947) this low figure is not surprising as the vigorous conditions probably caused some decomposition. No attempt was made to isolate the conjugated oestrogen as such, and in no case was more than 25% of the total administered oestrogen accounted for.

Mazur & Schorr (1942) dosed rabbits with stilboestrol and isolated from the urine 30% of the administered oestrogen as the monoglucuronide. No other synthetic oestrogens were investigated and no other metabolic products were reported.

We have found that in the rabbit a high percentage of the synthetic oestrogen given is excreted as glucuronide or other derivative soluble in sodium bicarbonate, while quite a small amount is excreted as free oestrogen. Much lower total excretion was found in the cat.

METHODS

Urine collection and extraction. The urines were treated as described by Simpson & Wilder Smith (1948), care being taken to avoid hydrolysis of glucuronide by heat or by bacterial growth. After acidification of the urine to Congo red with dilute HCl in the cold, ether extraction (by hand and continuous) yielded an extract which gave a fraction, soluble in bicarbonate, containing glucuronide, which was characterized as described by Simpson & Wilder Smith (1948), and a fraction soluble in caustic soda, containing free oestrogen. The latter extract was made acid to Congo red, extracted with ether and the extract assayed biologically, giving the yield of free oestrogen excreted. As an additional check, a sample of the original unextracted urine was tested for oestrogenic activity: this gave an indication of the combined activity due to free and conjugated oestrogen. After the sodium oestrogen glucuronidate had crystallized from the bicarbonate solution (where this was used) the mother liquor was acidified, extracted with ether, and the ether extract assayed biologically; this eliminated errors due to incomplete crystallization. That the ether extraction of the acidified but unhydrolyzed urine was complete was proved in several experiments by hydrolyzing afterwards, and extracting the urine with ether in a continuous extractor. No oestrogens could be detected either in the bicarbonate or caustic soda fractions from the ether extract. Thus complete extraction of both free and conjugated oestrogens had been attained (see below, p. 256).

Biological assay and expression of results. Usually the amount of oestrogen present in the urine was so great that the crude urine could be assayed without any preliminary extraction. In other cases the various fractions of the urine during extraction were assayed to serve as a check on the completeness of the chemical separations.

The method of assay was the one usual in this Institute: Spayed adult rats were injected twice daily for 3 days with the extract or urine to be tested and the vaginae of the rats were smeared twice daily during two following days. Smears consisting of epithelial and/or cornified cells only were taken to represent positive responses. The number of samples to be tested and the uncertainty of their activity made it impossible for more than 5–10 rats to be used at each dose level and the doses used were in the proportions 1, 2, 5, 10, 20, 50, 100, etc., so the results have only a very low degree of accuracy.

The content of the samples assayed biologically was estimated (on the assumption that all the activity was due to free oestrogen or to oestrogen glucuronide) by reference to dose-response curves for the substances concerned (Simpson & Wilder Smith, 1948). In either case and also where chemical separations were effected the content of free oestrogen was calculated and is expressed throughout as a percentage of the total dose given.

RESULTS

Elimination after subcutaneous injection into rabbits

The results summarized in Table 1 are derived from an experiment where four female rabbits were injected daily (except Sundays) with 30 mg. of dienoestrol or stilboestrol for 4 weeks.

The total proportion of the administered oestrogen eliminated is somewhat higher for stilboestrol than for dienoestrol. In this particular experiment the difference is not statistically significant but Stroud (1939) found the same difference, and in other experiments we have recovered as much as 46% of administered stilboestrol as the glucuronide.

During the injection period the total elimination remained fairly constant. There was a slight tendency for the proportion of free oestrogen to decline after the first week (though the third week in rabbit 3/46 is exceptional) and with stilboestrol there may be a slight delay before the glucuronide elimination process becomes fully effective. It may be noted that the crystallization of the glucuronide from the sodium bicarbonate was rather more complete with dienoestrol than with stilboestrol (cf. columns 4 and 5, Table 1).

One of the most striking facts revealed is that the elimination of these high doses is so rapid. Although the injections were made in oily solution and oil remained unabsorbed at the end of the sixth week of the experiment, yet even in the week after the injections had stopped the excretion had dropped to low levels.

Table 1. *Urinary elimination of subcutaneously injected oestrogens*

(Six injections of 30 mg. of oestrogen each week for 4 weeks.)

		Percentage of total weekly dose eliminated			
			(ii) As glucuronide		
Rabbit	Week	(i) Free	Gravimetric	Biological*	Total
		Dienoestrol injections			
No. 1/46, ♀	1	5	16	1	22
	2	1	—	—	—
	3	1	14	5	20
	4	1	15	5	21
	5	0·1	3	<1	3
	6	—	—	<1	<1
No. 2/46, ♀	1	5	21	1	27
	2	1	18	4	23
	3	1	17	4	22
	4	1	22	3	26
	5	0·1	—	<1	<1
	6	—	—	<1	<1
		Stilboestrol injections			
No. 3/46, ♀	1	10	1	10	21
	2	5	19	10	34
	3	15	7	10	32
	4	1	10	10	21
	5	0·1	—	>1	>1
	6	—	—	<1	<1
No. 4/46, ♀	1	10	2	10	22
	2	10	—	10	20
	3	5	10	10	25
	4	5	13	10	28
	5	0·1	—	4	4
	6	—	—	<1	<1

* Biological assay of mother liquids after removal of gravimetrically estimated compound.

Elimination after subcutaneous injection into cats

Two female cats each received 1·8 g. of dienoestrol (dissolved in sesame oil with a little added ethanol) by subcutaneous injection during 1 week. Their urine was collected during the week of the injections and the following week.

About 1 % of the injected oestrogen was excreted in the free form, but no glucuronide could be isolated. However, the fraction soluble in bicarbonate showed oestrogenic activity on biological assay, and if this activity were all due to glucuronide it would account for 4 % of the administered dienoestrol.

During the injection period the daily urine volume in the cats was abnormally low. A similar decrease had been noticed in the injected rabbits: in their case the decrease was due to the smaller consumption of cabbage, which was the chief component of their diet. It is well known that oestrogens in high doses depress the appetite and, although the fluid intake of the cats was not measured, we have little doubt that it was this which was responsible for the small output in the cats also.

The toxic effects of dienoestrol were much more marked in the cats than in the rabbits. Both the cats in this experiment died; one 2 weeks, and the other 3 weeks, after the first injection.

Effect of urinary pH *on elimination of injected dienoestrol by female rabbits*

A possible reason for the different rates of elimination of oestrogen glucuronide in cats and rabbits might lie in the difference in the reaction of their urine. That in the normally fed rabbit is alkaline while cats' urine is strongly acid.

To investigate this possibility two mature female rabbits were fed on a diet of oatmeal, bread and water until their urine was consistently acid to litmus (pH 5) and then injected subcutaneously with 1·5 g. of dienoestrol (dissolved in sesame oil) as before.

The results given in Table 2 show clearly that an acid urine does not prevent the elimination of large proportions of injected dienoestrol in the form of the glucuronide. Again, as with the experiments in rabbits with alkaline urine, the variations in glucuronide yield are wide.

Table 2. *Elimination of dienoestrol glucuronide in rabbits with acid urine* (pH 5) *after subcutaneous injection of dienoestrol*

	Dosage		Urine collection	
Rabbit	Time (weeks)	Amount (mg.)	Time (weeks)	Percentage of dienoestrol excreted as glucuronide
R 24/46	1–3	1500	1–7	24
R 25/46	1–3	1500	1–7	48

Table 3. *Elimination of hexoestrol glucuronide in rabbits after oral administration of hexoestrol*

		Days of		Percentage of hexoestrol excreted as glucuronide
Rabbit	Total dose (mg.)	(i) Dosing	(ii) Collection	
R 27/46	1000	1	1–9	30
R 31/46	700	1	1–4	46

Elimination after oral administration in rabbits

Single doses of hexoestrol, dissolved in aqueous ethanol or in sesame oil, were given by mouth to two female rabbits and their urine was collected (see Table 3).

The urine from R 27/46 was also assayed biologically. The activity found was equivalent to 5% of the administered dose if it were all free hexoestrol, or to 90% of the administered dose if it were all glucuronide. As this last figure is so much greater than the glucuronide isolated chemically it appears that some of the hexoestrol was, in fact, eliminated in the free form.

Similar experiments where two doses of stilboestrol, dienoestrol or hexoestrol were given 6 days apart gave similar results (Table 4).

Table 4. *Elimination of oestrogen after oral administration during* 6 *days of stilboestrol* (*S*), *hexoestrol* (*H*) *or dienoestrol* (*D*)

			Percentage of total dose eliminated			
				(ii) As glucuronide		
Rabbit	Total dosage (mg.)	Collection period (days)	(i) Free Biological	Gravimetric	Biological*	Total
R 32/47	1800 S	14	5	9	23	32
	Control†	14	<1	0	<1	<1
R 33/47	2000 H	11	<1	14	26	40
	Control	11	<1	0	<1	<1
R 30/47	1450 D	14	5	7	25	32
	Control	14	<1	0	<1	<1

* As in Table 1.

† Control collections were made after a lapse of 10 days from the end of the experimental collection.

In this experiment all the urine samples after ether extraction were hydrolyzed with hydrochloric acid under reflux and re-extracted with ether. These ether extracts were then worked up in the usual manner and biologically assayed. No oestrogenic activity was detectable in any of the extracts at the highest dose level tested (equivalent to a 1% recovery of the oestrogen given). This proves that all the conjugated and free oestrogen had been extracted from the unhydrolyzed urine in the first place. The results again show that the oestrogen eliminated in the urine is predominantly conjugated.

Urinary elimination of stilboestrol glucuronide fed to the rabbit

Stilboestrol glucuronide (250 mg.) dissolved in sesame oil was fed by stomach tube to a female rabbit, the urine being collected and worked up as in the previous experiments. The striking feature of the results (Table 5) is that very low yields of glucuronide were obtained, showing that the glucuronide is certainly not the end product of metabolism. A second experiment in which 100 mg. of stilboestrol glucuronide dissolved in sesame oil were injected subcutaneously yielded similar results (Table 5).

The results of this experiment demonstrate that the glucuronide formed after the administration of stilboestrol is not a stable end product but is further metabolized in the body.

Table 5. *Urinary elimination of oestrogen after single dose of stilboestrol glucuronide*

Rabbit	Dose (mg.)	Collection period (days)	Percentage of total dose eliminated			
			(i) Free	(ii) As glucuronide		
			Biological	Gravimetric	Biological*	Total
R 33/47†	250	4	1	12	1	13
R 7/47‡	100	4	3	0	25	25

* As in Table 1. † Oral administration. ‡ Subcutaneous injection.

DISCUSSION

Our results confirm those of Mazur & Schorr (1942) in demonstrating that rabbits excrete a high proportion of administered stilboestrol in the urine as glucuronide, but do not agree with the finding of Stroud (1939) that high proportions are excreted in the free form. This discrepancy is probably due to the method of working up used by Stroud. We have also found that dienoestrol and hexoestrol behave in the same way as stilboestrol, all yielding about 35 % of the administered dose as monoglucuronide in the urine. We have assumed that all the conjugated oestrogen is monoglucuronide as, in those cases where only 1 % remained for biological assay, the 99 % was in fact glucuronide. These conclusions relate to the very large doses we have used and are not necessarily applicable to lower, therapeutic doses.

The yields obtained in our earlier experiments were somewhat lower than those realized later; this is probably due to increased experience in handling these rather intractable substances. The main difficulty lies in the isolation of the glucuronides, either free or as sodium salts. This may be seen for example by the variable proportions of glucuronide remaining for biological assay in Tables 1, 4 and 5. Perhaps this can be attributed to the accumulation of various bicarbonate-soluble substances in the urine. If so, the time during which the urine is collected after dosage should be kept as short as possible, consistent with the recovery of the greater proportion of administered oestrogen; indeed the rapidity with which the dose is eliminated (see Table 1) renders any prolonged collection after dosage unnecessary.

The only stage so far identified in the metabolism of the synthetic oestrogens is that of glucuronide formation. We were unable to isolate any sulphate esters of the administered oestrogens and therefore have no direct evidence of their production in the rabbit. In man this form of elimination of natural oestrogens is well known and there is some indirect evidence of a small excretion of synthetic oestrogen sulphates (unpublished data).

It is very doubtful whether such metabolism studies can shed any light on the mechanism of oestrogenic action. Glucuronide and sulphate formation are too widespread to be specific for such action, while the complex degradation products of the natural oestrogens cannot be vital for oestrogenic action since the synthetic oestrogens produce the same effect without undergoing the same chemical changes. For closer study of oestrogenic action it would probably be advantageous to use the most chemically stable oestrogens available, e.g. hexoestrol (Wilder Smith & Williams, 1945, 1947) in order to avoid the complications of side reactions which are irrelevant to oestrogenicity.

SUMMARY

1. The urinary excretion of oestrogen after the administration of stilboestrol, hexoestrol, dienoestrol, and stilboestrol glucuronide has been examined in the rabbit and cat.

2. In the rabbit about 35 % of the administered oestrogen can be accounted for in the urine. In the cat much lower recoveries are obtained.

3. In the rabbit the chief metabolic products excreted are the monoglucuronides, but these substances are metabolized further to inactive products.

We are indebted to the Council of the Middlesex Hospital for the provision of laboratory facilities for this work and to Mrs Culliford and to Mr B. D. Shepherd for technical assistance. The investigation was aided by a grant from the Medical Research Council and by an apparatus grant from the Central Research Fund, University of London.

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The Isolation and Properties of the Monoglucuronides of Stilboestrol, Hexoestrol and Dienoestrol

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The monoglucuronides of stilboestrol, hexoestrol and dienoestrol were isolated from the urine of rabbits during metabolism experiments described previously (Wilder Smith & Williams, 1948). Mazur & Schorr (1942) described the glucuronide of stilboestrol which they obtained from rabbits treated with stilboestrol. As far as we are aware the glucuronides of hexoestrol and dienoestrol have not been isolated before.

METHODS

Urine collection and extraction. The urine was collected twice daily in the presence of toluene and stored at 0–3°. In the dienoestrol experiments a few mg. of hydroquinone were added daily to the collection vessels to assist stabilization.

After acidification to Congo red with dilute HCl the urine was extracted by hand three times with an equal volume of ether. Within limits, the larger the volume of ether used for each extraction, the less danger there was of the formation of intractable foams. The remaining urine was then extracted in a continuous ether extractor for 24 hr. and the combined extracts dried over anhydrous Na_2SO_4. After concentration to 20–60 ml. the ether solution was set aside in the refrigerator to crystallize.

In the method used by Mazur & Schorr (1942) the ether extract was not dried, but was extracted with saturated $NaHCO_3$ solution from which the sodium salt of the glucuronide crystallized. This method gave somewhat low yields with stilboestrol and dienoestrol and in the case of hexoestrol yielded no glucuronide at all (except on one occasion). The free glucuronide sometimes crystallized well directly from the ether extract when in sufficiently high concentration, but where difficulty was encountered with dienoestrol or stilboestrol the $NaHCO_3$ method was used. In cases where this expedient yielded no crystalline sodium salt, the bicarbonate solution was acidified to Congo red, extracted with ether, the ether removed and the residue taken up in a small amount of acetone. Addition of the acetone solution to saturated aqueous $NaHCO_3$ usually yielded the crystalline sodium salt. Solution of the crude sodium salts in water followed by acidification in the cold with dilute HCl precipitated the free glucuronides as a plastic mass. After washing with a small volume of water, and drying *in vacuo* over $CaCl_2$, the crude solid was dissolved in a little acetone and filtered. On adding excess dry ether to the filtrate a slight amorphous precipitate was formed which was filtered off. On keeping the filtrate at 0–3° the glucuronide slowly crystallized.

It was not found possible to remove a molecule of water of crystallization from the hexoestrol glucuronide even after drying for 6 hr. *in vacuo* at 110°. Dienoestrol and stilboestrol glucuronides also hold water of crystallization tenaciously, which is, however, almost completely removed under the above conditions.

Biological assay. The oestrogenic activity of the glucuronides was tested by subcutaneous and intravaginal administration to spayed rats. The technique used for the assay by subcutaneous injection has been described previously (Wilder Smith & Williams, 1948). The intravaginal assay was carried out by the application of two doses of 0·01 ml. of oily solution into the rat vagina 24 hr. apart; smears were taken during the two succeeding days. In both cases positive responses were indicated by vaginal smears consisting of cornified and/or epithelial cells only and dose response curves were constructed and the ED_{50} calculated by the method of Fisher & Yates (1943).

RESULTS

Chemical properties

Stilboestrol monoglucuronide. Mazur & Schorr (1942) report the melting point, analysis and optical rotation of this substance. We have found very similar properties, which are given here for confirmation. In addition the physiological properties are reported.

After recrystallization of the white needles several times from dry ether containing a trace of acetone and drying 2 hr. *in vacuo* at 110°, white, semi-fused needles were obtained, softening 100–110°, melting with decomposition 174–176°. After longer drying periods some samples yielded a pale yellow syrup. On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 10·7 mg. glucuronide lost 0·7 mg. (6·5 %) with extensive sintering. Theoretical loss for $1H_2O$, 3·9; for $2H_2O$, 7·5 %. On exposing the dried sample to the atmosphere for 48 hr. at room temperature, the glucuronide regained 0·3 mg. in weight. $[\alpha]_D^{24°} = -51·6°$ ($c = 1·60$ in absolute ethanol); $[\alpha]_D^{24°} = -57·6°$ ($c = 0·179$ in absolute ethanol). Equivalent weight = 451 (using 0·01 N-NaOH and methyl red as indicator). Theory = 444. Found: C, 64·1; H, 6·7. Calc. for $C_{24}H_{28}O_8$: C, 64·8; H, 6·4 %.

Hexoestrol monoglucuronide. This was obtained in the crude state by direct crystallization from an

ether extract of the acidified urine. Difficulty was experienced in the purification of this substance since it did not crystallize as the sodium salt from saturated aqueous sodium bicarbonate solution, except in one experiment where the concentration of glucuronide used was higher than usual. Further crystallization either from acetone and benzene or from acetone and ether mixtures was sometimes difficult. It was therefore purified by dissolving in sodium bicarbonate solution, extracting with ether, making the aqueous layer acid to Congo red paper with dilute hydrochloric acid, extracting with ether and drying over anhydrous sodium sulphate. The ether was removed by distillation, and the residue taken up in a small amount of acetone and reprecipitated by addition of ether. The slight amorphous precipitate first formed was removed by filtration, when the monoglucuronide crystallized from the filtrate slowly on standing at 0°. The crystals thus obtained gave a positive Tollens test (after 30 min. acid hydrolysis).

After drying the fine white crystals for 4 hr. at 110° *in vacuo* a white mass of partially fused needles sintering at 85–95° and decomposing at 188–196° was obtained. On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 7·9 mg. glucuronide lost 0·8 mg. (10·1 %) with some sintering. Theoretical loss for $3H_2O$, 10·8 %. On exposing the dried sample to the atmosphere for 48 hr. at room temperature the glucuronide regained 0·4 mg. in weight. The crystals were soluble in acetone and ethanol, sparingly soluble in ether, insoluble in light petroleum and benzene. $[\alpha]_D^{24°} = -73·4°$ ($c = 0·976$ in absolute ethanol). Equivalent weight = 482. Theory for the monohydrate of the monoglucuronide = 464. Found: C, 62·4; H, 6·7. $C_{24}H_{32}O_9$ requires C, 62·2; H, 6·9 %.

Dienoestrol monoglucuronide. This substance also proved difficult to purify since it did not crystallize well and was apparently somewhat unstable. The sodium salt of the glucuronide however crystallized from saturated aqueous bicarbonate solution and was used for purification. The free acid was soluble in acetone and ethanol, sparingly soluble in ether and insoluble in benzene and light petroleum and gave a positive Tollens test (after 30 min. acid hydrolysis). After drying 2 hr. *in vacuo* at 110° fine white needles were obtained, sintering at 105–115° and melting at 175–183° (with decomp.). On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 9·9 mg. glucuronide lost 0·1 mg. (1·0 %) with little sintering. On exposing the dried sample to the atmosphere for 48 hr. at room temperature 0·1 mg. was regained in weight. $[\alpha]_D^{24°} = -41·5°$ ($c = 0·560$ in absolute ethanol). $[\alpha]_D^{24°} = -47·2°$ ($c = 0·182$ in absolute ethanol). Equivalent weight = 426. Theory for monoglucuronide = 442. Found: C, 64·4; H, 7·0. $C_{24}H_{26}O_8$ requires C, 64·8; H, 5·9 %.

Table 1. *Oestrogenic activity of stilboestrol, hexoestrol and dienoestrol monoglucuronides in rats*

(Figures for free oestrogens given for comparison in brackets.)

Oestrogen	ED_{50} (in μg.) with 95 % fiducial limits: By subcutaneous injection in aqueous solution	By intravaginal application in oily solution
Stilboestrol monoglucuronide	5·8 (5·5–6·2)	0·04 (0·03–0·05)
[Stilboestrol	0·35	0·003]
Hexoestrol monoglucuronide	11·5 (8·7–15·3)	0·07 (0·04–0·10)
[Hexoestrol	0·35	0·003]
Dienoestrol monoglucuronide	7·2 (6·5–8·1)	0·15 (0·07–0·3)
[Dienoestrol	0·75	0·003]

Biological properties

The oestrogenic activities of the three glucuronides are recorded in Table 1. The activities of the corresponding free oestrogens are given for comparison. Although aqueous glycerol is the preferred solvent for intravaginal tests, arachis oil was used in the reported tests for the sake of uniformity.

DISCUSSION

All the glucuronides have only 5–10 % of the oestrogenic activity of the corresponding free oestrogen. This is true whether the assays are made by subcutaneous or intravaginal methods.

If the activity were due to the complete hydrolysis and liberation of free oestrogen then the glucuronides should show about 50 % of the activity of their parent compounds. In actual fact their activity is about 10 %, calculated on the weight of oestrogen present. The small activity which the glucuronides do show may be due either to their intrinsically low oestrogenic potency, to the liberation of only a proportion of free oestrogen or to more rapid elimination from the organism. That the proportion of free oestrogen liberated, if any, is very small indeed is indicated by the low excretion of free stilboestrol following the administration of its glucuronide (Wilder Smith & Williams, 1948), and is probably not sufficient to account for the biological

activity shown. From evidence so far available it is not, however, possible to formulate the factors to which the lowered potency of the glucuronides is due.

SUMMARY

The isolation and purification of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol, and their chemical and oestrogenic properties, are described.

We are indebted to the Council of the Middlesex Hospital Medical School for the provision of laboratory facilities for this work, to Dr A. C. Bottomley for help with the statistical side of the bioassays and to Mrs D. Culliford and Mr B. D. Shepherd for technical assistance and to the Medical Research Council for a grant.

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The Preparation and Some Properties of Hyaluronic Acid from Human Umbilical Cord

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Hyaluronic acid has now been known for ten years and in this period the literature about it has become both extensive and inconsistent. A number of substances with varying properties has been isolated by different methods from umbilical cord, skin, vitreous humour, synovial fluid, tumours and haemolytic streptococci. In spite of the differences in their properties, all are called hyaluronic acid if they give more or less viscous solutions and if they are composed for the most part of glucuronic acid and acetylglucosamine. There is no clear evidence whether several substances exist with somewhat similar properties, or whether the observed variations are due to contamination with unrelated material, or to partial destruction of originally homogeneous hyaluronic acid during the isolation.

As a prelude to an investigation of the effect of various agents on the enzymic decomposition of hyaluronic acid we have tried to define the conditions under which a product with more clearly defined properties can be prepared. This was necessary because crude preparations differed greatly in the rates at which they lost viscosity with testis hyaluronidase. Contamination of the preparation by substances activating or inhibiting the enzyme is the most obvious explanation of this phenomenon.

The elementary composition of a hyaluronic acid preparation is of little value as a criterion of purity. The likely contaminants have carbon and hydrogen contents similar to those expected for hyaluronic acid. The nitrogen content is a good index of heavy contamination with protein, but no great confidence should be placed on a 'correct' nitrogen value, for this is easily attained by the simultaneous presence of comparable amounts of protein and a nitrogen-free polysaccharide. There is substantial agreement that hyaluronic acid contains neither phosphorus nor sulphur but freedom from sulphur has not often been achieved in making preparations of hyaluronic acid.

Apart from proximate analysis, hyaluronic acid can be examined for its constituents: glucosamine, glucuronic acid and acetic acid; and the equivalent weight can be determined by titration with alkali. These measurements have not often been made on the preparations used as substrate in studies on hyaluronidase. Where they have been made considerable variation is apparent, especially in the case of determinations of the equivalent weight. This value should be 379 for the free acid, on the assumption that the molecule is so large that the ends may be neglected, but Meyer, Smyth & Dawson (1939) and Meyer & Chaffee (1940) have given a series of values ranging from 400 to 640, and our own values are within this range. It seems likely that the carboxyl group in at least part of the glucuronic acid is not free. The existence of this type of linkage robs the equivalent weight of some of its value as a criterion of purity.

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Viscosity has been widely used as an index of purity and of the extent to which degradation of the hyaluronic acid has been avoided during the preparation. This is legitimate if we assume that the native acid, in all the various sites where it occurs, has the same original viscosity, for many treatments are known which reduce the viscosity but none that increase it.

In Table 1 published values for the viscosity of preparations of hyaluronic acid are collected. It is clear that there is no uniformity in these values, and it is interesting to note that some of these preparations have been described by their producers as undegraded although the viscosity comes low in the list. For comparison the values for fractions made by the methods to be described in this paper are included in the same table.

Table 1. *Comparison of the viscosities of various hyaluronic acid preparations*

Source	Ionic environment	Temp. (°)	Concentration (g./l.)	Relative viscosity	Relative viscosity reduced to 1 g./l.	Reference
Cords	0·15 M-NaCl	20	2·5	9·3	4·3	Meyer, Smyth & Dawson (1939)
Cords	0·067 M-Phosphate buffer, pH 7	20	1·0	4·3	4·3	Blix & Snellman (1945)
Streptococci	0·15 M-NaCl	21	1·0	3·8	3·8	Seastone (1939)
Cords	0·1 M-Citrate-phosphate buffer, pH 7	30	3·8	10·0	3·4	Hale (1944)
Synovial fluid	0·067 M-Phosphate buffer, pH 7	20	1·0	2·55	2·55	Blix & Snellman (1945)
Cords	0·66 M-NaCl	34	1·0	2·0	2·0	McClean & Hale (1941)
Vitreous humour	0·15 M-NaCl	21	1·0	1·9	1·9	Seastone (1939)
Vitreous humour	0·067 M-Phosphate buffer	20	1·0	1·85	1·85	Blix & Snellman (1945)
Cords	Borate, chloride, phosphate, acetate mixture; 0·34 M in all, pH 7	25	2·3	2·0	1·4	Haas (1946)
Pleural exudate	0·15 M-NaCl, neutral	20	1·8	1·54	1·3	Meyer & Chaffee (1940)
Vitreous humour	0·5 M-NaCl	25	2·5	1·4	1·16	Madinaveitia & Quibell (1940)
Cords	0·083 M-NaCl, 0·083 M-citrate buffer, pH 4·5	25	16·5	2·6	1·1	Madinaveitia & Stacey (1944)
Cords	0·05 M-NaCl, 0·05 M-phosphate buffer, pH 7	25	1·0	8·2	8·2	This paper

The viscosities given in Table 1 were measured at different temperatures, at different concentrations, and in different ionic environments; part of the variation is due to these factors. At low salt concentrations the viscosity is high and it is greatly affected by slight variation in the salt concentration, but in the range in which most of the values fall the effect of changes in salt concentration is small. It is well known that the increment in the viscosity of a salt solution due to the presence of hyaluronic acid is not proportional to the concentration of hyaluronic acid but that the curve steepens as the concentration increases. These two factors have been disregarded in compiling the last column in Table 1 so that an approximate comparison of the various preparations can be made. In each case a value for the relative viscosity* at the standard concentration of 1 g./l. has been derived from the relation

$$1+\frac{\text{relative viscosity}-1}{\text{conc. at which viscosity was measured}}.$$

This approximation tends to exaggerate the viscosities of those products that were measured at the higher concentrations, but no better method of getting comparative figures seems to be available. With most of these preparations the curve relating relative viscosity to concentration has not been given, and Blix & Snellman (1945) have found that its slope varies from preparation to preparation.

The technique of fractionation used in this work was tried in order to test the basic idea that 'hyal-

* The terminology used in recent papers has not always been consistent. Here relative viscosity means the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration. Relative viscosity − 1 is a measure of the increase in viscosity due to the hyaluronic acid. It is sometimes called the specific viscosity, but we prefer to call it the viscosity increment.

uronic acid' is a name for a family of substances differing slightly in architecture and greatly in particle size. It would seem that the other workers in the field have tried to remove as much protein as possible and have then precipitated all the more or less viscous material in one fraction in spite of its apparent heterogeneity. In this work the appearance of viscous material in several fractions has not been looked on as a disadvantage; the fractions differ in their properties so much that nothing would be gained by mixing them. There is no satisfactory method of separating a mixture of protein and hyaluronic acid in one operation when the two are present in similar amounts. Digestion with commercial trypsin is not permissible if one intends to use the hyaluronic acid for studies on hyaluronidase because the protease preparations we have used inactivate hyaluronidase; there can be no certainty that separation of the inactivator from the hyaluronic acid has been complete. Several of the preparations referred to in Table 1 were made by removing the protein by shaking with chloroform and amyl or butyl alcohol. This process is very satisfactory for the removal of small amounts of protein in the final stages of a preparation, but the operations are tedious if used at the beginning. We prefer to precipitate most of the protein along with only a small part of the hyaluronic acid as a mucin clot and then to isolate the main part of the hyaluronic acid by the methods to be described.

EXPERIMENTAL

Preparation of hyaluronic acid

Human umbilical cords have been used as starting material in all our work. They are washed carefully and then preserved in acetone. After lying for 1–6 weeks in acetone they are cut up into pieces not more than 1 cm. long and extracted for a few more days with fresh acetone. The acetone is then drained off as completely as possible and the cords are extracted with successive lots of water, using about four times the cords' wet weight of water for each extraction and leaving each one for at least 2 hr. The first two extracts contain little but acetone and may be thrown away, but the later extracts bring out some hyaluronic acid accompanied by much protein. These extracts therefore give a mucin clot on acidification; they are combined and extraction is continued until the amount of clot is small. Five to eight extractions may be necessary. The combined extracts are taken to pH 3 and the clot collected so that it can be worked up for hyaluronic acid along with other fractions grossly contaminated with protein.

The residue is passed through a power-driven meat grinder with $\frac{1}{4}$ in. holes in the plate. To reduce the viscosity the ground material is suspended in 3 vol. of approx. 0·1 M-NaCl, and after some hours it is poured into a cloth and the fluid is expressed by hand. The extraction is repeated twice, and the new NaCl solution is allowed each time to lie in contact with the cord for at least 12 hr. If this work is not carried out in a cold room the fluids should be kept saturated with chloroform at all times. For each litre of the combined extracts 20 ml. of approx. 5 N-HCl are added with thorough stirring. If the initial washing of the cords was adequate there will be only a small precipitate; this is stirred gently and intermittently during some hours, until it coalesces into a string which is then removed and added to the mucin clot fraction.

Hyaluronic acid can be precipitated from this fluid in several different ways. The conventional methods of precipitation with several volumes of ethanol or acetone are inconvenient because of the bulk of the fluid involved. 100 g. dry weight of washed cords (about 40 cords) give 6 l. of extract at this stage. Two alternative precipitation procedures are described.

(*a*) $(NH_4)_2SO_4$ (300 g.) are added to each litre of the clear acid fluid. Purified hyaluronic acid is not precipitated by $(NH_4)_2SO_4$, but the residual protein in the solution precipitates and brings out some hyaluronic acid with it. The precipitate that separates entangles air forced out of the solution by the salt; this is convenient for it is easier to allow the mixture to stand till all the solid has collected at the top, then to siphon off the subnatant fluid and compact the solid scum by centrifuging, than to de-aerate and centrifuge down the precipitate. To each litre of fluid 50 ml. of pyridine are added and the mixture is shaken vigorously by hand; some of the pyridine remains undissolved in the $(NH_4)_2SO_4$ solution and a little solid separates at the interface. The mixture is allowed to settle, the clear fluid is siphoned off and the interfacial material is compacted by centrifuging. This centrifuging should be done in a covered tube so that the pyridine does not evaporate, because the pellicle remains insoluble only if some pyridine phase is present. The pellicle generally contains little hyaluronic acid. A further 250 g. of $(NH_4)_2SO_4$ are now added. This forces more pyridine out of solution and it brings with it hyaluronic acid which stays at the interface between the two liquids. On centrifuging in closed vessels, it becomes a compact coherent sheet that can be lifted out of the centrifuge tube with a pair of forceps. Approximately equal amounts of hyaluronic acid are present in the original $(NH_4)_2SO_4$ precipitate and in the second pyridine pellicle, but the former may contain as much as 20% of protein and the latter only 5% judging from the nitrogen content. In each case the protein can be removed by shaking with chloroform and amyl alcohol according to the Sevag technique.

(*b*) A comparable method for separating hyaluronic acid from the acid cord extract involves precipitation with acid ethanol in the presence of $(NH_4)_2SO_4$. It offers no advantages over the method already described and involves handling larger volumes of fluid, but there may be other sources of hyaluronic acid with which it might have advantages. Ethanol (1·33 vol.) is added to the acid fluid; a fine gelatinous precipitate separates and this is entangled with air bubbles. The whole precipitate may be sufficiently coherent to float up in 1 hr., thus enabling one to siphon away most of the fluid. If it fails to do this, the air is removed from the precipitate by exposure to vacuum for a few minutes. The precipitate will now sink and can be centrifuged down. This precipitate is suspended evenly in water and reprecipitated by addition of 1·33 vol. of ethanol; on dialysis it goes into solution slowly as the ethanol is removed. Solid $(NH_4)_2SO_4$ is added to the ethanolic supernatant fluid with vigorous mechanical stirring. It dissolves

slowly and about 200 g. are needed to saturate 1 l. On standing, the system separates into two layers; the lower contains much $(NH_4)_2SO_4$ and little ethanol and the upper little $(NH_4)_2SO_4$ and much ethanol. As a rule a precipitate which is relatively uncontaminated hyaluronic acid collects on the interface. When separation has proceeded so far that both liquid layers are clear, as much of each as possible is siphoned off and the interfacial material collected by packing it into a coherent felt by centrifuging. It packs very tightly when centrifuged and should be diluted with water before dialysis, for otherwise a rigid jelly will be formed that dialyzes slowly. The proportion in which these two fractions appear varies from preparation to preparation; there is always an ethanol precipitate, but sometimes little or no material separates on the addition of $(NH_4)_2SO_4$. Residual protein is removed from these fractions, as from the pyridine fractions, by the Sevag technique. For effective removal of protein from these viscous solutions, the concentration must be kept low. Solutions of crude hyaluronic acid containing less than 2 g./l. at pH 4 in the presence of 0·1–0·5 M-salt are shaken briskly by hand with a mixture of one part chloroform to two of amyl alcohol, and then centrifuged. The aqueous solution is shaken again, this time mechanically, with fresh solvent. As a rule little or no emulsion is made on this second shaking. After acidification with HCl the fluid can be either dialyzed directly or precipitated with 1·33 vol. of ethanol and then dialyzed in a smaller volume of water. In the latter case precipitation will be incomplete, but this is an advantage because the unprecipitated material has a lower viscosity than that precipitated. It can be recovered as an interfacial precipitate by adding $(NH_4)_2SO_4$.

The yield of purified material, by either of the two methods, is 1–2% of the dry weight of the cords. The cord residue and the mucin clots that have been precipitated by adding acid to the extracts contain more hyaluronic acid, and this can be recovered, in a less viscous form, by digestion with proteolytic enzymes. The cord residue, of a consistency such that little more can be squeezed out of it by hand, is suspended evenly in about 5 vol. of 0·01 N-HCl containing 0·5 g./l. pepsin. The pH is readjusted to 2 if it is significantly higher than this, and the mixture is incubated with toluene for 24 hr. at 37°. The greater part dissolves leaving a pink solution and a curd. These are easily separated by filtration on a coarse paper at room temperature. Sufficient gelatin is liberated into the fluid to

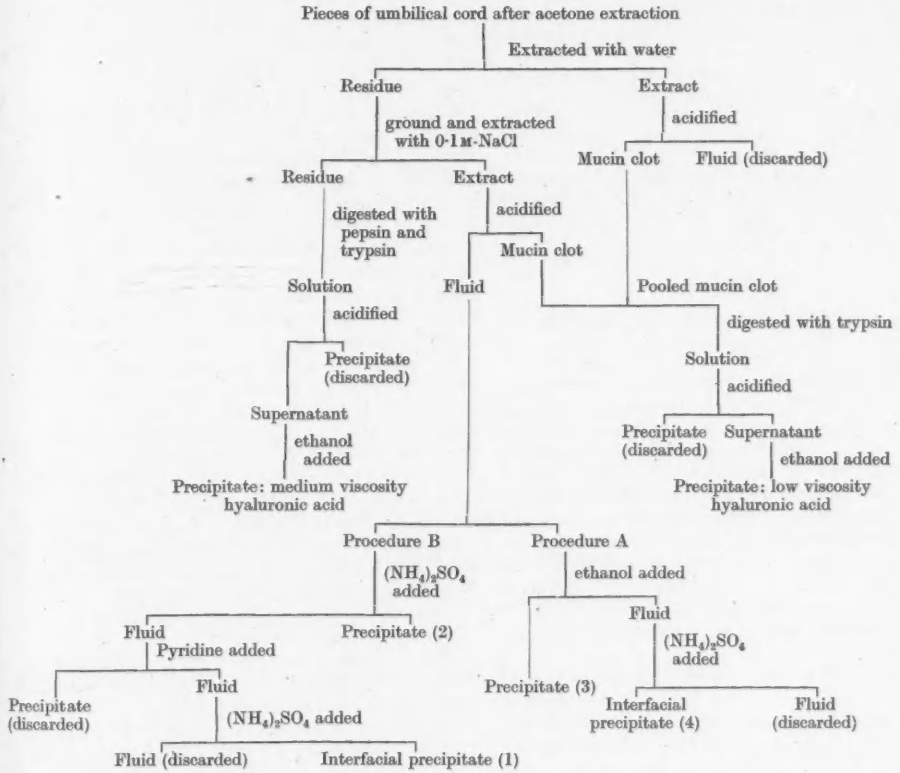

Fig. 1. Processes used in the extraction of hyaluronic acid in different forms. Precipitates (1), (2), (3) and (4) are shaken with chloroform and amyl alcohol to give hyaluronic acid fractions with variable but high viscosities.

cause gelatinization if it is cooled. This pepsin extract gives an ethanol precipitate and an interfacial precipitate in much the same way as the extract from the minced cords, but each has a high nitrogen content. It is preferable therefore to neutralize the pepsin extract and digest it with trypsin to destroy the protein that it contains. The cord residue is also digested with trypsin and the two extracts are combined. These digestions are carried out at 37° in the presence of toluene with 2 g./l. commercial trypsin for 24 hr. at pH 7·6. When these trypsin-digested fluids are put through the acid-ethanol-$(NH_4)_2SO_4$ fractionation, nothing precipitates with the conc. HCl and very little interfacial material separates. What there is, therefore, is added to the ethanol precipitate and this precipitate is dissolved, reprecipitated, and dialyzed.

The mucin clot can be partially fractionated by precipitation with ethanolic potassium acetate in alkaline solution as in McClean's (1943) method of purification, but it is generally more satisfactory to incubate it with trypsin and then work up the hyaluronic acid by ethanol precipitation in the manner just outlined for the pepsin extract of the cord residue. The yields of these two products are variable, but that from the pepsin extract may be as high as 4%, and that from the mucin clots 1%, of the dry weight of the cords. It is clear, therefore, that our methods for the preparation of the viscous fractions of hyaluronic acid lead to incomplete extraction in the first place and to significant losses during the purification. This state of affairs seems to be unavoidable if viscous preparations are desired. Material from the pepsin extract has only half the viscosity increment of the viscous fractions and that from the mucin clots is even less viscous. Slightly more viscous products can be made from these by precipitating a series of fractions with ethanol or ethanolic potassium acetate, but no product has been made from them at all resembling the viscous fractions. In Fig. 1 an outline is given of the processes used in making these various fractions, and also of the sequence in which they occur.

Properties of preparations of hyaluronic acid

General

Umbilical cords that have been washed with acetone and water and dried contain 2·5–3% of amino sugar estimated as glucosamine by the method of Elson & Morgan (1933). This amount of glucosamine would correspond to 6–8% of hyaluronic acid, which is equal to the total yield that we get. The non-viscous fractions however contain less than the theoretical percentage of glucosamine. Some of the glucosamine is therefore unaccounted for, but it is clear that the greater part of the glucosamine of the cord is present in the form of polysaccharides that can be isolated by the methods described here.

Preparations made from the acid-soluble part of the original cord extract are all clear and colourless, those made by digesting the mucin clot or the cord residue are yellow or pink. The nitrogen content (Kjeldahl) lies in the range 2·8–4·3%. The phosphorus content is lower than 0·05%, but sulphur contents of 0·5–1% are usual. The form in which this sulphur occurs is not known. Viscous preparations contain between 9 and 11% of acetyl, but preparations made by enzymic digestion of the residues have lower acetyl contents, sometimes as low as 6%. The acetyl content was measured by hydrolyzing a sample for 75 min. at 100° in 2·5N-H_2SO_4, steam distilling in the apparatus described by Markham (1942) and titrating the distillate with N/70 NaOH. There is a similar deficiency in the glucosamine content; non-viscous preparations contain 20–25%, whereas viscous preparations contain 34–38%. These values are in the range found by most other workers. Glucosamine determinations were made by the Elson & Morgan (1933) technique on material that had been hydrolyzed for 6–8 hr. at 100° with 5N-HCl and then evaporated to dryness in a vacuum desiccator over NaOH. These analyses were made on portions of dialyzed solutions, the concentration of which was determined by drying at 100° for 1–2 hr., and cooling in a desiccator. They refer to the free acid and not to a salt.

Viscosity

Viscosity measurements were made in Ostwald viscosimeters with capillaries 9 cm. long and having flow times of about 30 sec. for 4 ml. of water. This volume was used in all the experiments reported here and, except where another temperature is specified, all measurements were made at 25°. Viscosity measurements made in the absence of salt are of little significance; the large influence exerted by low concentrations of salt makes such a measurement an index of the completeness of dialysis rather than of the state of aggregation of the hyaluronic acid. From a consideration of the results of Madinaveitia & Quibell (1940) and of our own results we have adopted 0·05M-NaCl and 0·05M-phosphate buffer at pH 7·0 as the standard ionic environment for measurements. Small variations in salt concentration in this region have little effect on the viscosity. Much of the work on hyaluronic acid has been done at pH 7, and we adopted this pH because we intended to use it in subsequent enzyme studies. A more acid pH would be in some ways preferable, for the viscosity of hyaluronic acid in 0·025M-citrate buffer and 0·15M-NaCl, is unaffected by the pH in the range 4·5–7·3. There is a sharp fall in viscosity to about half the maximum between pH 4·5 and 2·6. Above pH 7·3 there is a more gradual fall to 95% of the maximum value at pH 8·5.

Pyridine precipitates, before the removal of residual protein by shaking with chloroform and amyl alcohol, are the most viscous products that we have encountered. Solutions containing 0·3 g./l. have a relative viscosity in salt of 2·2–2·5, but this falls to 1·9–2·2 when the protein is removed. The results of Blix & Snellman (1945) suggest that this may be due to atmospheric oxidation during the shaking; it

might be avoided, as they suggest, by shaking in nitrogen but we have not tried this. The three other viscous products, after removal of protein, have relative viscosities of 1·7–2·0 for the 0·3 g./l. solution. In these cases the removal of protein may be accompanied by either a rise or a fall in viscosity. Occasional batches have had viscosities lower than this, and there is a gradual fall in viscosity as solutions age. Products from the cord residue have relative viscosities of 1·3–1·5 at 0·3 g./l. and those from the mucin clot 1·01–1·15. McClean & Hale (1941) and Blix & Snellman (1945) have published data on the non-linearity of the relationship between viscosity and the concentration of hyaluronic acid solutions. Our results are in agreement; with viscous preparations an increase in the concentration from 0·3 to 0·6 g./l. increases the viscosity increment three- to fourfold, and solutions containing 1 g./l. have a relative viscosity of more than 8. With less viscous preparations the relationship is practically linear.

Effects of temperature on the viscosity

Differences in the temperature at which the measurements were made must contribute to the differences in the results collected in Table 1. The flow times of 0·3 g./l. solutions of preparations covering a range of viscosities have been measured at various temperatures in the salt mixture already specified. The flow time of all is reduced by approximately one third between 20 and 37°, but much of this reduction is due to the change in the viscosity of the buffer solution. Over this temperature range the change in viscosity increment is never more than 15 % of the value at 20°. Temperature is clearly not a significant factor in explaining the differences between products made in different laboratories.

Effect of different salts on the viscosity

Madinaveitia & Quibell (1940) and McClean & Hale (1941) have shown that the viscosity of hyaluronic acid depends on the ionic environment. We have tried the effect of several salts on different preparations, and the results shown in Fig. 2 are typical of those given by viscous preparations from which the protein had been removed by the Sevag technique. An abrupt fall in viscosity with traces of salt is followed by a more gradual fall to a minimum viscosity at about 0·5 M. There is then a gradual rise in relative viscosity; the values at 1 M are approximately the same as those at 0·01 M.

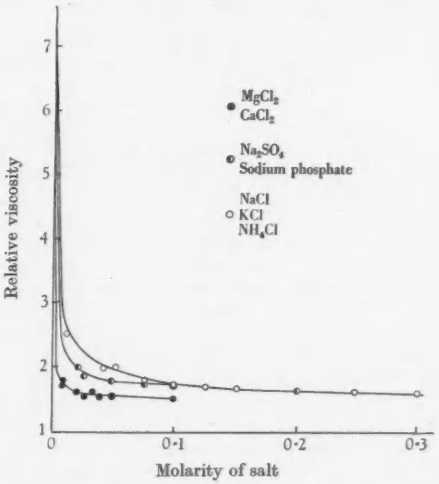

Fig. 2. Change in the viscosity of hyaluronic acid solution with varying concentrations of different salts. Temp. 25°; pH 7; 0·3 g./l. solution of a medium-viscous preparation.

Calcium and magnesium chlorides have clearly a greater effect than the other salts, but no generalizations can be made on the basis of such a small number of salts.

SUMMARY

1. Methods are described for preparing hyaluronic acid from human umbilical cords.

2. By preparing a series of fractions some products have been made with a greater viscosity than any hitherto described.

3. Some data on the chemical and physical properties of these fractions are given.

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The Effects of Serum and of Hyaluronic Acid Derivatives on the Action of Hyaluronidase

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In the preceding paper (Hadidian & Pirie, 1948) the viscosity of hyaluronic acid preparations in a defined environment was used as a criterion in selecting standard preparations. In this paper the loss of viscosity by such a preparation has been used to follow the course of hyaluronidase action. This method has the primary advantage that loss of viscosity appears to be the first change that can be recognized during the enzymic decomposition of hyaluronic acid. Factors influencing this *in vitro* change are more likely, therefore, to affect the action of hyaluronidase *in vivo* than those influencing the later stages of the decomposition of the molecule. It is clear that several enzymes are involved in the hydrolysis of hyaluronic acid to reducing sugars. Thus Hahn (1945) has effected a partial separation of the enzyme responsible for oligosaccharide production from that responsible for monosaccharide production; and Rogers (1946*b*) finds differences between the products of the reaction catalyzed by enzymes from different sources. He interprets this as evidence that the enzymes studied by Hahn are present in different ratios in these sources and points out that it is improbable that either enzyme brings about the reduction in viscosity.

Several inhibitors are known for the action of hyaluronidase on hyaluronic acid, whether this is measured by fall in viscosity, mucin-clot prevention or appearance of acetylglucosamine. McClean & Hale (1941) and Hobby, Dawson, Meyer & Chaffee (1941) found that the sera of animals, that had been injected with hyaluronidase from different sources, inhibited the action of the enzyme used for immunization but did not inhibit the action of other hyaluronidases. Later, however, McClean (1942) mentioned briefly an inhibitor present in normal serum, and this substance has also been investigated by Haas (1946). Heparin, gastric mucin, chondroitin sulphuric acid and partly depolymerized hyaluronic acid also inhibit the enzyme. McClean (1942) suggests that these glucosamine-containing substances act competitively because of their structural similarity to hyaluronic acid. Working on this suggestion we have made some inhibitors by modifying hyaluronic acid. We also record some observations on the inhibitor present in serum.

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EXPERIMENTAL

General

Viscosity measurements were made by the technique used in the preceding paper (Hadidian & Pirie, 1948). The time taken for the viscosity to fall half way from the initial to the presumed final value has been taken as a measure of the rate of enzyme action. This measurement of 'half-time' is conventional in studies on this enzyme and has been adopted for convenience, although it is clear that it has no particular merit. It does not, for example, correspond to the loss of viscosity by half of the hyaluronic acid present but to a smaller proportion than this; and to a proportion that varies inversely with the original viscosity of the hyaluronic acid. This is due to the non-linearity of the relation between the concentration and the viscosity of a hyaluronic acid solution, and to the non-linearity of the relation between the axial ratios of elongated particles and the viscosity of their solutions. The enzyme action we are studying can be pictured in two extreme ways. The enzyme may attack a particle of hyaluronic acid and proceed to break linkages in that particle until the products have little or no effect on the viscosity of the solution; the half-time would correspond to a state where less than half the particles have been affected because of the first non-linearity. On the other hand, it may break all the particles to some extent before any one particle has been so far reduced as to have little effect on the viscosity of the solution; because of the second non-linearity the half-time would correspond to the breaking of less than half the relevant linkages.

Some confusion is also introduced into the use of half-time measurements in studying inhibition reactions by the viscosity of the inhibitors. In many cases the viscosity of the inhibitor is unaffected by the enzyme; the final value for the end of the action can therefore be taken as the viscosity of the inhibitor solution in the salt mixture used. In some cases, however, the sum of the increments in relative viscosity, due to hyaluronic acid and inhibitor separately, is less than the observed increments when they are present together. This effect, when it occurs, is small and as long as no significance is attached to differences in half-time less than 30%, it is unlikely to alter the conclusions.

Measurements of the rate of loss of viscosity have been made on an extensive range of hyaluronic acid preparations under identical conditions and with the same enzyme preparation. The half-times have varied by a factor of ten. Part of this variation is due to differences in the initial viscosities of the preparations. If differences in viscosity are due to differences in the average length of the particles in a preparation, the half-time corresponds to the breaking of a smaller number of linkages when the particles are long than when they are short. The generalization that the more viscous the preparation, the shorter the half-time, is not,

however, found to be invariably true. This has suggested to us that umbilical cord may contain substances that activate or inhibit the action of hyaluronidase on hyaluronic acid, and that these may have been removed to varying degrees during the purification. The fact that viscous preparations are the most uniform in their half-time is in favour of this interpretation. We have tested many of the products discarded during the purification and have found, in agreement with other workers, that fractions of very low viscosity are inhibitors. No fraction giving activation has been consistently preparable.

Preparation of inhibitors

Nitration of hyaluronic acid and substances related to it. Dry hyaluronic acid, with the loose, open structure it has after drying by sublimation, is cooled in a freezing mixture to about $-10°$. Sixty parts of fuming HNO_3 (sp.gr. 1·59), also cooled to the same temperature, are added and the mixture is maintained at $-10°$ for 15 min. with occasional stirring. All the hyaluronic acid dissolves in a few minutes. Ice (300–400 parts) is now added with assiduous stirring. The temperature falls still further and a white curd separates from a brilliant blue fluid. As soon as most of the ice has melted and all pockets of undiluted HNO_3 in the curd have been broken up with a rod, the mixture is centrifuged for a few minutes and the solid is washed 3–4 times on the centrifuge with ice water. It is kneaded carefully each time to get rid of as much HNO_3 as possible, and the washing is continued until the wash water can be neutralized by a drop or two of 0·1 N-NaOH. The solid is now suspended in distilled water and brought into solution by adding sufficient NaOH to bring the pH to 6–7. This pale yellow, viscous fluid will be referred to as acid-insoluble nitrate.

All the supernatant fluids are mixed and kept cold. They are neutralized with 5 N-NaOH with vigorous stirring and cooling so that the temperature never rises above 20° during the neutralization. The fluid, at pH 6, is concentrated on a hot plate in a draught of air until $NaNO_3$ begins to crystallize out. It is then dialyzed for 10–20 hr. to remove most of the nitrate, concentrated again, and redialyzed. At this stage the solution from 100 mg. of hyaluronic acid has a volume of 2–3 ml. The solution remaining after thorough dialysis will be referred to as acid-soluble nitrate.

The ratio of acid-soluble to acid-insoluble nitrate varies from preparation to preparation. When hyaluronic acid of low viscosity (e.g. with a relative viscosity of 1·4 for a 1 g./l. solution measured in the presence of salt) is used as the starting material there is little acid-insoluble product. When even less viscous fractions are used there is no acid-insoluble material at all. It seemed possible, therefore, that all the acid-soluble material was derived from the less viscous components of an initially non-homogeneous hyaluronic acid. It is unlikely that this is the sole origin; for if the acid-insoluble nitrate is dried by sublimation and nitrated again by the method described, a further quantity of soluble nitrate is formed. Some degradation of the molecule during nitration is to be expected, and this explanation of the origin of some of the soluble nitrate is borne out by the increased proportion of product in that form if the nitration is continued for longer than 15 min. The sum of the weights of nitrate in both forms is about equal to the starting weight of hyaluronic acid and, when highly viscous hyaluronic acid is used, 85% is in the acid-insoluble form.

Nitration always leads to some fall in the viscosity of hyaluronic acid. The extent of this fall is variable, but in general the viscosity increment of the acid-insoluble nitrate is about half that of the parent hyaluronic acid. The acid-soluble nitrate is much less viscous. Nitrated hyaluronic acid, of both types, can be salted out from neutral solution by $(NH_4)_2SO_4$. With the acid-insoluble product, precipitation begins at about one tenth of saturation and is complete at one third of saturation. The more readily precipitated fractions are the more viscous, and they are precipitated fully by lower concentrations of $(NH_4)_2SO_4$ than the less readily precipitated fractions. It is clear, therefore, that the nitrate is a non-homogeneous mixture, but it is not certain whether this reflects an original non-homogeneity of the parent hyaluronic acid or is due to destruction during the nitration. Precipitation of the acid-soluble nitrates and of nitrates derived from hyaluronic acid preparations of very low initial viscosity does not start until one third saturation by $(NH_4)_2SO_4$. Salting out does not therefore seem to be a useful alternative to neutralization, evaporation and dialysis in the preparation of acid-soluble nitrates because, although acid-insoluble nitrate is precipitated by saturated $NaNO_3$, the soluble nitrates are not, and the high nitrate concentration confuses $(NH_4)_2SO_4$ precipitation.

Hyaluronic acid is not salted out by $(NH_4)_2SO_4$ from solutions containing no other component, but, as one of the methods suggested for the purification shows, it can be salted out if pyridine or some forms of protein are also present. The nitrates behave similarly, for, although esterification of the hydroxyl groups has made the nitrates sufficiently hydrophobic to be salted out, they can be salted out at lower concentrations of $(NH_4)_2SO_4$ in the presence of pyridine.

There is no reason to doubt that the hyaluronic acid has nitrated as other polysaccharides do (cf. Speiser & Eddy, 1946); for the dry product burns in a flash and the nitrogen content (Dumas) has risen from 3–4 to 8·6%. No estimate other than the measurement of nitrogen content has been made of the number of nitrate groups introduced. If hyaluronic acid has the structure generally assigned to it, a chain of alternate glucuronic acid and acetylglucosamine residues connected by glucoside linkages, there are four free hydroxyl groups for each repeating disaccharide unit. The sodium salt of the tetranitrate that could be made from such a substance would contain 12% of nitrogen and that of the dinitrate 8·55%. Esterification of the hydroxyl groups in this way does not apparently affect the *N*-acetyl group, for the acetyl content falls from 10–11 to 7–8%. The value to be expected for the dinitrate is 8·7%. Control experiments show that neither nitrous nor nitric acids, in amounts comparable to those present in a hydrolysate, are estimated as volatile acid after hydrolysis and distillation by the procedures used on these samples. On the other hand direct estimation, by the Elson & Morgan (1933) method, on the nitrated material shows an apparent disappearance of the glucosamine. This result is due to the action of the HNO_3 liberated during hydrolysis. The same disappearance can be brought about by hydrolyzing normal hyaluronic acid in the presence of a quarter of its weight of $NaNO_3$, an amount corresponding to four nitrate groups. Furthermore, de-esterification, by the method to be described later, restores the apparent glucosamine content, and hydrolysis in the presence of a reducing agent such as HI protects the glucosamine to a great extent from destruction. Fuming HNO_3 probably acts to some extent as an oxidizing as well as a nitrating agent, but it is clear that no oxidation involving

the glucosamine is going on to an extent at all comparable to that found by Skanse & Sandblad (1943) to accompany peroxide oxidation.

Acetylation of hyaluronic acid

Like some other acid polysaccharides (e.g. alginic acid, pectin and agar) hyaluronic acid does not acetylate readily. Acetic anhydride in neutral or alkaline aqueous solution, or in the presence of pyridine under anhydrous conditions, failed to bring about significant acetylation in a few hours at room temperature or on slight warming. Two recent methods are, however, effective; in one the acid is swollen with acetic acid and H_2SO_4 is used as catalyst (Chamberlain, Cunningham & Speakman, 1946), and in the other the acid is swollen with formamide and acetylated with acetic anhydride and pyridine (Carson & Maclay, 1946). The products of these two acetylations differ somewhat in their properties.

(1) Hyaluronic acid is swollen with acetic acid by adding glacial acetic acid in a thin stream to a 3–10 g./l. solution. Mixing is complete until about 2 vol. have been added, after which precipitation begins and is substantially complete with 4 or 5 vol. The mixture is centrifuged and the solid washed with acetic acid and used immediately. This precipitation does not lead to any significant fractionation of the hyaluronic acid, for only about 3% of the starting material is found in the solution when this is evaporated to dryness. This soluble fraction has the normal nitrogen and glucosamine content, but its viscosity is low. The precipitate, on the other hand, if dialyzed immediately, gives a solution with the original viscosity. We do not, therefore, agree with the statement made by many workers on hyaluronic acid that precipitation with acetic acid leads to a fall in viscosity; but our observations are in agreement with those of Meyer, Smyth & Dawson (1939) who prepared a relatively viscous product from synovial fluid by acetic acid precipitation.

The fibrous clot of hyaluronic acid is pressed as free from acetic acid as possible and added to 200 times its weight of a mixture of 1 part of conc. H_2SO_4, 90 parts of acetic anhydride and 120 parts of benzene. There is little or no solution of the hyaluronic acid, and, after a suitable interval, the mixture is centrifuged and the solid suspended in water and dialyzed. If acetylation is allowed to proceed for a few minutes only, the product has an acetyl content of 16–20%; after 40 min. at 22° this rises to 25–33% and is not increased by longer acetylation at that temperature. By following the method of Chamberlain *et al.* (1946) and raising the temperature to 60° for 15 min., after acetylation has proceeded for 1 hr. at 25°, the acetyl content can be raised to 36%. Acetyl determinations were made by the method used in the preceding paper (Hadidian & Pirie, 1948) and include both *O*-acetyl and *N*-acetyl. The theoretical value for the tri-*O*-acetyl derivative of a polysaccharide built up from equal quantities of glucuronic acid and *N*-acetyl glucosamine is 34% and for the tetra-*O*-acetyl derivative 39·4%. It would therefore appear that, as in the case of nitration, esterification remains incomplete. Products that have been acetylated for a few hours only are still soluble in water over the whole pH range, but those that have been acetylated at room temperature for many hours, or have been heated to 60° during acetylation, are not soluble on the acid side of neutrality. There is a progressive fall in viscosity during acetylation. Products acetylated for a few minutes only retain 70–80% of the original viscosity increment, whereas the tri-*O*-acetates retain only 10% or less.

(2) Hyaluronic acid that has been dried by sublimation does not change its appearance when left for a few hours at 20° in formamide, but at 40° it swells somewhat and at 50° turns in 1 hr. to a viscous dough when suspended in ten times its weight of formamide. This dough can be mixed with an equal volume of pyridine by stirring for a few minutes. The plastic, rather than fluid, mixture is now mixed with half its volume of acetic anhydride, which causes an initial shrinkage, but is incorporated into the mixture in a few minutes. As in the other method of acetylation the properties of the final product depend on the duration of acetylation and the temperature at which it proceeds. The mixture is stirred with three times its weight of ice; when it is brought to pH 2 with 5N-HCl a soft curd separates. The fluid is dialyzed. If acetylation has proceeded for 1 hr. at 20° this acid-soluble product has an acetyl content of 14–16%, while after 3 hr. at 30° it rises to 30%. In neither case has it any significant viscosity. The curd is not soluble at neutrality in the absence of salt and is only partly soluble in the presence of salt, but it swells slowly to give a suspension of gelatinous particles, which dissolve at pH 9–10. The solutions have only one fifth of the viscosity of the parent hyaluronic acid. All these acetylated fractions coagulate with strong acid and, like the nitrates, can be precipitated with $(NH_4)_2SO_4$.

De-esterification. The nitrates and acetates of hyaluronic acid can be partly de-esterified without extensive degradation by exposure to alkali in the presence of ethanol. Solutions of either ester containing 3–10 g./l. are mixed with 10 vol. of ethanol. With the esters, unlike with hyaluronic acid itself, no precipitate separates even on the addition of a salt such as potassium acetate. The addition of ethanolic KOH at 22° leads to the appearance of an opalescence, and later, after a time interval depending on the amount of alkali added, of a precipitate. For example, an ethanolic solution containing 0·8 g./l. of a moderately viscous nitrate (rel. visc. 2·3 at 1 g./l. in salt) gave an immediate precipitate with 7 g./l. KOH; but with 2·5 g./l. precipitation took 2 hr., and with 0·7 g./l. precipitation occurred only on the addition of some salt. Similarly with the acetate there is immediate precipitation with 1–2 g./l. KOH, but with less than this amount precipitation is delayed. Under these conditions de-esterification remains incomplete as determined from the nitrogen and acetyl content of these products. The immediate purpose of the work, the making of substances that inhibit hyaluronidase is, however, satisfied; and conditions that would lead to complete de-esterification have not been investigated.

Factors affecting the hyaluronidase-hyaluronic acid reaction

Effect of salt concentration on the rate of reaction. In studying the effect of possible inhibitors and activators on the hyaluronidase-hyaluronic acid reaction, it would first be necessary to establish conditions of salt concentration under which such work can be carried out with the least possible interference from salt effects. Results of Robertson, Ropes & Bauer (1940), McClean & Hale (1941), and

Madinaveitia & Quibell (1941) show that there is an optimum salt concentration for the reduction of the viscosity of hyaluronic acid by hyaluronidase, but the optima they find vary with their different substrate and enzyme preparations. We have tried the effect on this reaction of several salts, previously studied for their viscosity-reducing activity, with results shown in Fig. 1. These reactions were carried out at pH 7·0, using salt solutions buffered with 1% of phosphate or borate buffer. Of necessity, pH control in low salt concentrations was not quite adequate, but frequent checks showed that variation was not more than 0·2 unit; this may produce as much as 20% variation in the rate of reaction. From the results shown and from others on different substrates, three groups are distinguishable: $CaCl_2$ and $MgCl_2$, effective in very low concentrations; NaCl, KCl and NH_4Cl, just as effective but in higher concentrations; Na_2SO_4 and Na_2HPO_4, relatively ineffective. Sodium acetate is intermediate between the chloride and phosphate. Lineweaver & Ballou (1945), in their study of the effect of salt concentration on the activity of pectin esterase, find similar variations in the effectiveness of various salts in activating the enzymic reaction, but with that enzyme there is the same maximum activation at the optimum concentration of each salt. We agree with McClean & Hale (1941) that the chloride ion is an important activating agent. However, the difference in the effectiveness of the chlorides cannot be attributed to Cl^- alone. Expressed as normality of Cl^-, assuming complete ionization, the optima with Mg^{++}, Ca^{++}, Na_4^+, NH^+ and K^+ are at 0·05, 0·06, 0·1 and 0·15 respectively. Furthermore, in the presence of 0·05 M-phosphate, maximum activation is obtained with 0·05 M-NaCl. Two factors seem to be involved; general salt concentration and the presence of an activating ion, such as Cl^-. The pH optimum in the presence of 0·05 M-NaCl and 0·05 M-phosphate is at 6·3; this value is intermediate between the optima of 6·8 and 5·0 found by McClean (1943) in 0·017 M- and 0·167 M-NaCl respectively. Our experiments have been carried out at pH 7 as a compromise between the pH optimum and the pH at which the enzyme and inhibitors are used in *in vivo* tests that will be described elsewhere.

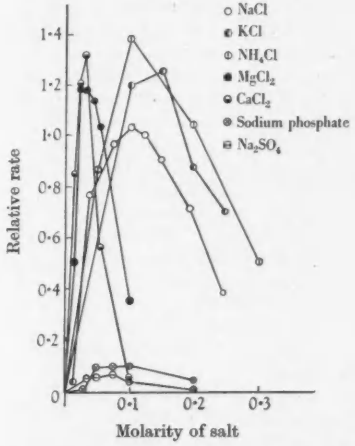

Fig. 1. Variations in the rate of hydrolysis of hyaluronic acid by hyaluronidase with varying concentrations of different salts. Temp. 25°; pH 6·9±0·10; 12·5 mg./l. bull-testis enzyme; 0·3 g./l. solution of a medium viscous hyaluronic acid preparation. In the experiments with NaCl, KCl, NH_4Cl and Na_2SO_4 the pH was maintained with phosphate buffer, and in those with $MgCl_2$ and $CaCl_2$ with borate buffer. In all cases the molar ratio of salt to buffer was 100. In expressing the relative rates, the reciprocal of the half-time in 0·125 M-NaCl is taken as unity.

Estimation of inhibitory activity. Since no method exists for direct estimation of hyaluronidase, the course of the reaction between it and an inhibitor has to be followed by the indirect method of determining the amount of hyaluronidase that is made unavailable for the hyaluronic acid-hyaluronidase reaction in the presence of the inhibitor or after the hyaluronidase has been exposed to the action of the inhibitor for a given period of time. If, then, one works in a region where there is a linear relationship between hyaluronidase concentration and the rate of loss of viscosity by hyaluronic acid, one can justifiably consider the rate as a direct measure of the amount of active hyaluronidase in the system. The range over which such a relation exists varies with different preparations of hyaluronic acid tested at the same concentration. With highly viscous preparations and with the partially purified enzyme preparation that we use, deviation from linearity is slight up to an enzyme concentration of 8 mg./l. With less viscous preparations this range may extend up to 50 mg./l. The following methods are used in estimating inhibitory action: in instances where the length of exposure of the enzyme to the inhibitor is of little or no consequence, the enzyme is introduced into a mixture containing both inhibitor and substrate, and the subsequent viscosity changes followed; where the extent of inhibition is dependent on the duration of exposure of the enzyme to the inhibitor, as in the case of inhibition by serum, the enzyme and the inhibitor are incubated together under the proper salt and pH conditions for a given length of time, then the substrate is introduced into the mixture, and the course of the reaction followed. The extent of inhibition is determined from the ratio of the half-time of the reaction with inhibitor to that without. Thus a threefold inhibition would be obtained if a half-time of 600 sec. is obtained with inhibitor as compared with 200 sec. without. The reciprocal of this ratio is the fraction of the initial enzyme concentration that is active. Partially purified bull-testis enzyme (Schering) was used in all these experiments.

Inhibition by hyaluronic acid esters. Nitrates and acetates that have been made from viscous hyaluronic acid retain much of their initial viscosity. The effect of hyaluronidase on them can therefore be

tested in the usual way. The viscosity of nitrates is not reduced by hyaluronidase and the viscosity of acetates often falls at first and then stays constant. We look on acetates behaving in this way as mixtures of material that can be attacked and material that is not attacked and does not inhibit the reaction. The stability of these hyaluronic acid esters towards the enzyme contrasts with the susceptibility to attack of the *p*-aminobenzyl ether (Humphrey, 1943). In Humphrey's preparations, however, less than one ether linkage seems to be present for each glucosamine in the hyaluronic acid, and no indication is given of the extent to which hydrolysis, which was recognized by the appearance of *N*-acetylglucosamine, took place.

with nitric esters are more systematic; all of them inhibit. Attempts to get less nitration by using a smaller ratio of nitric acid to hyaluronic acid or by allowing the nitration to proceed for a shorter time have led to less active products. The acid-soluble fractions from the nitration of viscous material and the acid-soluble nitrates of non-viscous material are stronger than the acid insoluble nitrates. By suitably controlled removal of nitrate groups with alkali and ethanol the inhibitory power is substantially increased; but it is clear from the table that the intensity of the alkali treatment is of importance and that too drastic a treatment leads to a weaker inhibitor. For comparison the results of experiments with non-viscous hyaluronic acid and

Table 1. *Effects of various inhibitors on the hyaluronidase-hyaluronic acid reaction*

Description of inhibitor	Concentration (g./l.)	Inhibition
Viscous hyaluronic acid acetylated by sulphuric method for 60 min.; 33% acetyl	0·1	1·5
Above product deacetylated for 15 min. with 2 g. KOH/l.; 14% acetyl	0·1	6
Viscous hyaluronic acid acetylated by sulphuric method for 10 min.; 18% acetyl	0·1	4
Viscous hyaluronic acid acetylated by formamide method for various times; 15, 25 or 30% acetyl	0·1	Inactive
Acid-soluble product from nitration of viscous hyaluronic acid	0·01	3·5
Acid-insoluble part of the same nitration	0·1	7·3
Acid-soluble product from nitration of viscous hyaluronic acid	0·03	3·0
Acid-insoluble part of the same nitration	0·03	2·2
Above insoluble product denitrated for 2 hr. with: 0·7 g. KOH/l.	0·005	5
2·5 g. KOH/l.	0·004	7·5
7·5 g. KOH/l.	0·03	2·2
Nitrated non-viscous hyaluronic acid	0·01	5
The same non-viscous hyaluronic acid before nitration	0·1	2
Heparin	0·1	2

In these experiments 2 ml. of a 0·6 g./l. solution of hyaluronic acid in 0·1 M-NaCl and 0·1 M-phosphate buffer pH 7 were mixed with 1 ml. of a solution of the inhibitor at four times the concentration specified in the second column. 1 ml. of a 50 mg./l. solution of hyaluronidase was then added and the flow time in an Ostwald viscosimeter was measured at various intervals. The half-time was found in the usual way, and the amount of inhibition set out in the third column is derived by dividing the half-time found in the presence of the inhibitor by that found in a control experiment with enzyme and substrate alone.

The inhibition is measured by mixing suitable amounts of inhibitor with hyaluronic acid and then adding the enzyme. There is a slight increase in the inhibition if the enzyme and inhibitor are mixed first, but in this instance the extent of inhibition is not increased by allowing the enzyme and ester to be in contact for more than the time necessary for mixing. Some comparable results are collected in Table 1. Values are given for only one concentration of each inhibitor because the inhibition is proportional to the concentration. The table shows that acetates inhibit weakly, or not at all, and the most active are partially acetylated products the preparation of which is a matter of some uncertainty. On partial de-acetylation the inhibition always increases, but conditions that would lead to regularly repeatable results have not been found. The results

heparin are also included in the table. Many non-viscous preparations of varied origin have been tested and none has been found with a significantly greater inhibitory power than the one cited. Only one sample of heparin was tried; the inhibition found here is comparable to that found by McClean (1942) and Rogers (1946*a*) using different experimental procedures. Our tests were made at 25° with hyaluronic acid having a relative viscosity of 7–8 for a 1 g./l. solution in 0·05 M-NaCl and 0·05 M-phosphate buffer pH 7, although experiments described in the next paragraph show that there is more complete inhibition with smaller concentrations of phosphate. The same sample of dried, partially purified hyaluronidase from bull testes (Schering) was used throughout at 12·5 mg./l. Similar experiments have been made with larger and smaller concentrations of

enzyme, at 37° and with samples of hyaluronic acid having a relative viscosity as low as 2 for the 1 g./l. solution. These variations in the conditions have not significantly affected the amount of inhibition observed.

Variations in phosphate and NaCl concentration, however, have a marked effect on inhibition by the esters. Several of these, including an acid-soluble nitrate, an acetate, a denitrated nitrate and a de-acetylated acetate, were tested in 0·05M-NaCl with phosphate concentrations varying between 0·013 and 0·24M; and in 0·05M-phosphate with NaCl concentration varying within the same range. Within this range there is an increasing inhibitory activity with decreasing salt concentration. None shows a significant inhibitory activity in 0·05M-NaCl and 0·24M-phosphate and all show maximum activity in 0·05M-NaCl and 0·013M-phosphate; but they all follow different courses between these extremes. Products with identical inhibitory activity under our standard testing conditions may differ by a factor of 2, at lower phosphate concentrations. Thus the figures given in Table 1 would not necessarily indicate the relative effectiveness of these substances under physiological conditions.

For convenience of writing, the operations that have been performed on hyaluronic acid in making these inhibitors have been called acetylation, nitration and de-esterification. It is certain that the bulk of the material is undergoing these changes, for the observed acetyl content can be increased and diminished as can the Dumas nitrogen content and the inflammability of the nitrates. It is less certain that these are the changes primarily responsible for the inhibition. All of them are associated with changes in viscosity and so presumably in particle size, and it is possible that this is the significant change rather than the extent of esterification. The difficulty found in getting reproducible results from experiments on acetylation and de-acetylation slightly favours this interpretation. On the other hand, the stability of these inhibitors in the presence of hyaluronidase suggests that a graded breakdown of the molecule is not alone sufficient to make a good inhibitor. Furthermore, our attempts to degrade the molecule by other means, e.g. acid, alkali and partial enzyme digestion, have resulted in only feebly active products. It is probable that inhibitors have both an optimum particle size and an optimum degree of substitution. The high activity found with nitrated non-viscous products suggests that this size may be fairly small; but active material does not diffuse through cellophan.

Serum as an inhibitor. McClean (1942), in the course of studying the *in vivo* decapsulation of streptococci, discovered a factor in the sera of several species which inhibits enzyme from different sources, and noted that they varied greatly in their inhibitory activity. He considered the possibility that this inhibition is due to the presence in the serum of a polysaccharide which acts by competing with the substrate for the enzyme. Haas (1946), on the basis of some by no means conclusive evidence, has claimed that this inhibitory action of serum is due to the presence in it of an enzyme which inactivates hyaluronidase.

The inhibition of hyaluronidase by serum at 25° proceeds at a measurable rate, as shown by Haas (1946). The method used for its study by Haas, and with some modification by us, has been to expose the enzyme to the action of serum for a given length of time, then mix it with hyaluronic acid solution containing enough phosphate buffer to obtain a final concentration of 0·05–0·1M-phosphate. The validity of this method rests upon the fact, discovered by Haas and confirmed by us, that in the presence of such concentrations of phosphate (the exact concentration varies with different serum samples) the activity of hyaluronidase in reducing the viscosity of hyaluronic acid is unaltered by incubation with serum. One cannot, however, assume on such evidence that interaction between hyaluronidase and the serum factor ceases in the presence of phosphate.

If one introduces phosphate into a mixture of hyaluronidase and serum that has been incubated for a time sufficient to inactivate a part of the hyaluronidase, and then removes samples at various intervals for testing their hyaluronidase content, one finds that in low phosphate concentrations (0·01–0·05M) the inhibition of hyaluronidase will continue, and in high phosphate concentrations (0·05–0·1M) it will be reversed. However, at about 0·05M the rate of change in either direction is very slow. Since the process of estimating the hyaluronidase after the inactivation reaction seldom lasts longer than 30 min., this method of studying the inhibitory action of serum seems valid so long as significance is not attached to small changes in activity.

Like the reaction between hyaluronidase and hyaluronic acid, the reaction of dialyzed serum with hyaluronidase does not proceed in the absence of salt. It is activated by small amounts, with an optimum between 0·03 and 0·1M-NaCl. The exact optimum varies somewhat with the amount of serum used, but variations within the range specified are slight. In 0·2M-NaCl the inactivation of hyaluronidase by serum at the end of 10 min. of incubation is about one fifth of the maximum. Phosphate will activate this reaction to a lesser extent. However, it is much more effective relatively in this reaction than in the hyaluronidase-hyaluronic acid reaction. In the presence of 0·025M-phosphate the inhibition attains 75% of the maximum obtainable in NaCl. With higher concentrations of phosphate, as of NaCl,

the serum-hyaluronidase reaction is apparently inhibited, 0·075 M-phosphate giving full inhibition. The serum used in these experiments was dialyzed for 4–5 days against several changes of distilled water. For the most part we have used pig serum in this study, but most of the experiments described have been repeated with human, rabbit, chicken or bovine serum. In view of the statement of Haas (1946) that serum obtained by clotting is less active than that obtained from blood defibrinated by stirring, all sera used were obtained in the latter manner. However, in subsequent experiments with sera obtained by clotting and by stirring from the same specimen of blood, we have been repeatedly unable to demonstrate under optimal salt conditions any significant difference between the two.

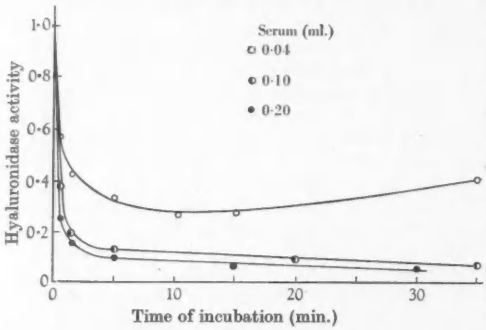

Fig. 2. Rate of inactivation of hyaluronidase by varying amounts of serum. 0·04, 0·1 and 0·2 ml. dialyzed pig serum, pH 7; incubation at 25° with 50 μg. of testis enzyme in 2 ml. of 0·05 N-NaCl containing 0·3 ml. borate buffer pH 6·9. 2 ml. of 0·6 g./l. solution of a high viscosity hyaluronic acid in 0·1 M-phosphate buffer and 0·05 M-NaCl added to the serum-hyaluronidase mixture at the end of incubation and the residual hyaluronidase activity determined viscosimetrically. Hyaluronidase activity $= \frac{\text{half-time without inhibition}}{\text{half-time with inhibition}}$. Owing to the non-linearity of the relationship between enzyme concentration and the rate of hydrolysis of this particular hyaluronic acid preparation the actual amount of hyaluronidase, expressed as fraction of the original amount remaining active after incubation is about a third more than the activity indicated.

As a result of these findings, the following procedure was adopted for testing the inhibitory activity of serum, with such modifications as were required by varying conditions. The enzyme (50 μg.) is incubated with 0·05–0·3 ml. of dialyzed, neutralized serum for 10 min. in 2 ml. of 0·1 M-NaCl buffered with 0·2 ml. of 0·2 M-borate buffer at pH 6·9. To this is then added 2 ml. of hyaluronic acid solution, 0·6 g./l. in 0·1 M-phosphate, and the course of the depolymerization of hyaluronic acid followed viscosimetrically.

Having established beforehand the range of enzyme concentration over which the relationship between hyaluronidase concentration and the rate of loss of viscosity by the hyaluronic acid preparation to be used does not deviate too markedly from linearity, one can follow the course of apparent inactivation of the enzyme by the serum. At 25° with 50 μg. of enzyme and 0·10 ml. of dialyzed pig serum in 2 ml. of buffered 0·05 M-NaCl, only about 50 % of the enzyme remains active 30 sec. after mixing, and 25 % after 1 min. (Fig. 2). Maximum inhibition is reached within 10–500 min. depending upon the serum concentration. With high concentrations of serum for the next 5–10 hr. no apparent change occurs, but eventually, in every instance the hyaluronidase activity begins to return (Fig. 3).

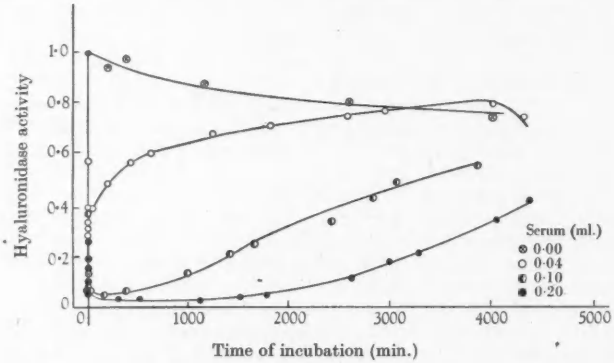

Fig. 3. Recovery of hyaluronidase activity after its inhibition by varying amounts of serum. Conditions of experiment are the same as specified under Fig. 2, where the course of the reaction for the first 35 min. is shown in detail. Serum incubated under the same conditions, but without hyaluronidase, had lost none of its inhibitory activity at the end of the experiment when tested on a fresh enzyme solution.

When there is no excess of serum, with 0·04 ml., recovery of hyaluronidase activity immediately follows its maximum inhibition. When there is an excess, apparently a condition of equilibrium is reached and is maintained as long as there is an excess of the serum factor available. The recovery process in every instance is slow, and is complete within 72 hr. only with low concentrations of serum. A hyaluronidase solution kept under the same temperature, salt and pH conditions may lose a quarter of its activity during this period. Serum kept under identical conditions loses very little, if any, of its original activity. In repeated experiments, the final hyaluronidase activity was never more than that at the beginning of the experiment, thus making it unlikely that this phenomenon is due to the production of hyaluronidase by contaminating bacteria. Experiments were also carried out in the presence of 0·05 M-NaF and 0·05 M-benzoate to prevent any possibility of bacterial contamination. Both interfere to some extent with hyaluronidase activity but the reappearance of hyaluronidase after inhibition can be demonstrated in their presence.

In these experiments inactivation and re-activation proceed in a solution that is not otherwise altered. By changing the environment the re-activation process can be hastened. Dilution of the mixture without change in salt concentration causes a 15 % increase in the rate for fourfold dilution. The same dilution doubles the rate of re-activation if it is made with NaCl containing 0·03 M-phosphate and quadruples it if made with NaCl containing 0·1 M-phosphate.

We find the reaction between serum and hyaluronidase much faster than is manifest from the data of Haas (1946). This in part is due to the fact that Haas uses no salt in his reaction mixture except borate buffer and whatever salt there is present in the serum, in part to the fact that he has no determinations at less than 5 min., and in part to his presentation of the course of the reaction as an increase in the 'activity' of serum. Thus, according to his data, 0·1 ml. chicken serum has an 'activity' of 2 at the end of 5 min., and 4 at 20 min. The former corresponds to an inhibition of 65 % of the hyaluronidase, and the latter to 80 %, i.e. only an additional 15 % of the original hyaluronidase is inhibited between 5 and 20 min. How much of the 65 % inhibition at the end of 5 min. takes place in the first few seconds there is no way of determining from Haas's data, but from our own data it is evident that the reaction is slowed abruptly after about 1 min. when an excess of serum is present.

The inhibition of hyaluronidase by derivatives of hyaluronic acid resembles the serum inhibition, in a general way, in its dependence on the ionic environment, although the serum inhibition is suppressed by concentrations of phosphate at which all esters tested have retained some activity. The actions differ in two significant respects; ester inhibition reaches its maximum more quickly than serum inhibition and there is no recovery on prolonged incubation. It is obvious that the hyaluronic acid esters cannot be called enzymes, but the terminology most suitable for the serum inhibitor is uncertain. Haas (1946) has called it an enzyme because the reaction proceeds at a measurable rate and because the inhibitor is destroyed at 55°, but these properties, which we confirm, are not exclusively the properties of enzymes and the fact that the inhibited hyaluronidase can recover its activity is a strong argument against the applicability of the word enzyme to the inhibitor. Essentially, an enzyme is a substance that combines with its substrate in such a way that, when dissociation occurs, the substrate is altered; the serum inhibitor combines with hyaluronidase and dissociates from it without affecting the only property of hyaluronidase of which anything is known. Under these circumstances the analogy would seem to be with the antigen : antibody and toxin : antitoxin reactions rather than with the enzyme : substrate reactions. The indiffusibility of the inhibitor and its inactivation by heating suggest that it is, or that it is associated with, a protein and this is borne out by McClean's observation, which we confirm, that it precipitates in the globulin fraction of serum. The destruction of the inhibitor on incubation with a hyaluronidase preparation could be due to a protease present in the enzyme, but it is also possible that it is another manifestation of hyaluronidase activity. On this view the inhibitor is a substrate for hyaluronidase on which the enzyme acts slowly; it is this that distinguishes it from the esters for they are apparently not attacked at all by the enzyme. In an attempt to get evidence for this point of view we have estimated the glucosamine in hydrolysates of the ultrafiltrate from dialyzed serum that had been incubated with hyaluronidase for sufficiently long to destroy a part of its inhibitory power. No glucosamine could be detected. This suggests that the inhibitor does not contain glucosamine, that this is not liberated during its destruction, or that the quantity is too small for detection by our methods. There is therefore still no evidence on the nature of the inhibitor or the mechanism of its action.

SUMMARY

1. The effect of various salts on the action of hyaluronidase on hyaluronic acid has been studied viscosimetrically. There is an optimum molarity for each salt, but its value and the rate of the reaction at the optimum vary with different salts.

2. Methods of preparing nitrates and acetates of hyaluronic acid are given. These esters inhibit the

enzyme action and this inhibition is compared with that brought about by a constituent of normal serum.

3. The extent of the inhibition by these agents is dependent on the salt concentration.

4. The serum inhibitor, unlike the inhibitors made by esterifying hyaluronic acid, is destroyed by the testicular hyaluronidase used.

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The Fate of Certain Organic Acids and Amides in the Rabbit

3. HYDROLYSIS OF AMIDES BY ENZYMES *IN VITRO*

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In the first two papers of this series it was shown that the rabbit is able to hydrolyze the carbamyl group in benzamide and phenylacetamide (Bray, Neale & Thorpe, 1946), but that the introduction of a hydroxyl group in the position *para* to the carbamyl renders the latter stable, less than 7% hydrolysis occurring *in vivo* (Bray, Ryman & Thorpe, 1947). Further investigations are in progress of the effect of hydroxyl groups in other positions and also of amino and nitro groups. This paper reports a parallel study in which we have examined the activity of various enzyme preparations, especially those from rabbit liver, in hydrolyzing the carbamyl group.

Similar investigations are those of Gonnermann (1902, 1903), in which the action of several enzyme preparations, including pepsin, trypsin and sheep's liver and kidney, on a number of amides and anilides was studied, of Geddes & Hunter (1928) and of Grassmann & Mayr (1933) who examined the action of yeast asparaginase on several amides. The criterion used by Gonnermann as an indication of hydrolysis was the isolation of the unchanged amide or of products of its hydrolysis, whilst Geddes & Hunter estimated the ammonia liberated. Relevant results from these investigations are summarized in Table 1. Geddes & Hunter claim that asparaginase is present in calf liver and also that yeast asparaginase will hydrolyze glutamine. Glutaminases hydrolyzing this amide but not asparagine have been shown to be present in various tissues, including liver and kidney (Krebs, 1935).

In the present work we have determined quantitatively the degree of hydrolysis by a formol titration method based on that used by Balls & Lineweaver (1939) for assessing the activity of papain with hippurylamide as substrate. We have also compared the stability of the amides used to acid and alkaline hydrolysis.

METHODS

Materials

Amides. The amides which could not be purchased were prepared by the action of aqueous ammonia upon the corresponding acid chloride or upon the methyl ester of the parent acid. Aminobenzamides were prepared by the reduction of the nitrobenzamides with ferrous sulphate (Jacobs & Heidelberger, 1917).

Table 1. *Summary of results of some previous investigations of enzymic hydrolysis of amides*

(Results marked * due to Geddes & Hunter (1928). Results marked † due to Grassmann & Mayr (1933). Unmarked results due to Gonnermann (1902).

	Amide						
Action of	Formamide	Acetamide	Propionamide	Benzamide	Salicylamide	Asparagine	Glutamine
Pepsin	−	−	.	−	−	.	.
Trypsin	−	+	.	−	−	.	.
Ptyalin	−	−	.	−	−	.	.
Invertase	−	−	.	−	−	.	.
Maltase	−	−	.	−	−	.	.
Emulsin	−	+	.	−	−	.	.
Sheep's liver	+	+	.	+	.	.	.
Calf's liver	.	.	.	.	.	+*	.
Sheep's kidney	+	−	.	+	+	.	.
Yeast asparaginase	−*	−*†	−*†	.	−*	+*†	+* −†

Sand. British Drug Houses Ltd. acid-washed sand was found to give more satisfactory results than other preparations tried.

Buffers. The majority of the experiments were carried out at pH 7·4 in a phosphate buffer consisting of 0·2 M-Na_2HPO_4 (8 ml.) and 0·2 M-NaH_2PO_4 (2 ml.). Where other buffers were used details are given in the appropriate section.

Preparation of extracts

The rabbit was killed by a sharp blow on the back of the neck and immediately bled. The liver and kidneys were removed and weighed. The organs were then separately ground with sand and mixed with water (1·5 times the original weight). The resulting *brei* was centrifuged and the supernatant liquid used in the experiments, either directly or after dialysis for 24 hr. The extracts were preserved with chloroform.

Method of estimation of amide splitting

Liver or kidney extract (10 ml.) was added to 50 ml. of a 0·0174 M-solution of the amide in the appropriate buffer. The mixture was incubated at 37° in a stoppered flask. At suitable intervals 4 ml. samples were withdrawn, treated with 0·5 ml. neutralized 40% formaldehyde and 1 ml. 0·1 N-NaOH and centrifuged. Five ml. of the supernatant liquid were titrated against 0·02 N-NaOH using thymol blue, taking the first appearance of blue (pH 9) as end point. Thymol blue was more convenient than phenolphthalein owing to the reddish colour of the digests. With the nitrobenzamides, which are sparingly soluble, 0·00687 M-solutions were used. Complete hydrolysis of 4 ml. digest corresponds to a titre of 2·9 ml. 0·02 N-NaOH. The corresponding titre for nitrobenzamides is 1·14 ml.

Controls used were (i) the mixture as above, but with boiled extract, and (ii) the mixture without the substrate. The titration values of such controls remained steady throughout the experiments except in a very few cases when the titre of the control increased slightly (up to 0·3 ml. 0·02 N-NaOH). The indistinctness of the end point makes it doubtful whether titration differences of less than 0·1 ml. are of any real significance.

Calculation of results

Digests prepared as above using a 0·0174 M-solution of the ammonium salt of the acid instead of the corresponding amide gave results in close agreement with the theoretical values for complete hydrolysis. This theoretical increase in titre was used as the basis for calculation of the degree of hydrolysis.

RESULTS

Experiments with papain

The hydrolysis of benzamide, *p*-hydroxybenzamide and *p*-nitrobenzamide was compared with that of hippurylamide (Balls & Lineweaver, 1939). Digests were made up from 20 ml. of papain solution (3% papain (British Drug Houses Ltd.) in water) and 50 ml. of the amide (0·0174 M in acetate buffer pH 4·9). A control for each amide consisted of 20 ml. boiled papain solution with 50 ml. of the amide solution. A control of 20 ml. papain solution with 50 ml. buffer pH 4·9 was also included. Typical results are shown in Table 2 and Fig. 1. Complete hydrolysis in this experiment corresponds to an increase in titre of 2·47 ml. 0·02 N-NaOH (0·98 ml. in the case of *p*-nitrobenzamide). *p*-Hydroxybenzamide did not appear to be hydrolyzed at all; the increase in titre of *p*-nitrobenzamide was so small as to be of doubtful significance.

Experiments with liver extracts

Hydrolysis of benzamide. The degree of hydrolysis of benzamide was determined for each liver extract. Two typical results are shown graphically in Fig. 5. The following figures show the percentage hydrolysis of benzamide effected by 29 different liver extracts in 10 hr. (The figures in parentheses give the percentage hydrolysis at equilibrium.) 38 (47); 20 (58); 11 (44); 35 (44); 20 (32); 19 (44); 19 (46); 9 (34); 27 (77); 15 (53); 14 (25); 4 (40); 17 (52); 38 (60); 16 (37); 35 (38); 44 (47); 32 (44); 39 (62); 32 (45); 33 (38); 27 (35); 44 (55); 25 (52); 26 (49); 15 (51); 14 (40); 15 (33); 22 (32). It can be seen that there is considerable variation. The average values are 24% for 10 hr. and 45% at equilibrium.

Table 2. *Hydrolysis of* 0·0174 M-*solutions of hippurylamide, benzamide,* p-*hydroxybenzamide and* p-*nitrobenzamide* (0·00687 M) *by papain at* pH 4·9

Time (hr.) ...	0	0·5	1·0	1·5	2·5	3·75	4·0	5·25	6·75	24	28
	Titres in ml. 0·02 N-NaOH										
Hippurylamide	6·75	7·05	7·2	7·3	7·55	7·7	—	7·75	7·85	8·05	8·1
Hippurylamide control	7·95	—	—	—	—	—	7·6	—	—	7·6	7·65
Benzamide	6·6	6·75	—	6·8	6·85	—	—	6·9	—	6·9	—
Benzamide control	7·7	—	—	—	—	—	7·7	—	—	7·65	—
p-Hydroxybenzamide	8·05	—	—	—	8·0	—	—	7·95	—	8·05	—
p-Hydroxybenzamide control	8·3	—	—	—	—	—	8·35	—	—	8·45	—
p-Nitrobenzamide	6·6	6·7	6·65	—	6·65	—	—	6·75	—	6·75	—
p-Nitrobenzamide control	7·15	—	—	—	—	—	—	—	—	7·05	—
Enzyme + buffer	6·85	6·8	—	6·7	—	7·0	—	6·8	—	6·85	6·85

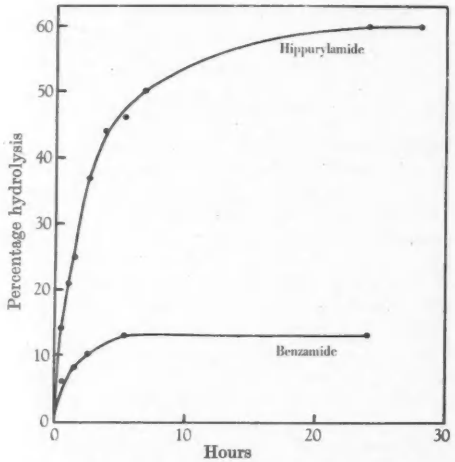

Fig. 1. Hydrolysis of 0·0174 M-hippurylamide and benzamide by papain at pH 4·9.

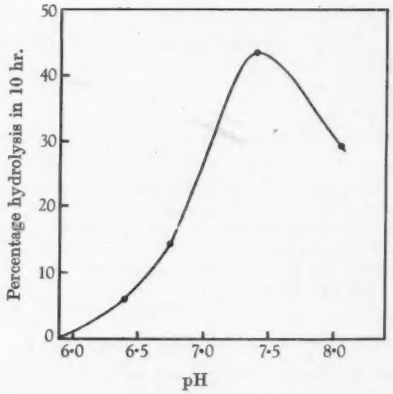

Fig. 2. Effect of pH on hydrolysis of benzamide by rabbit-liver extract.

Effect of pH *on activity of liver extracts.* Several experiments of which the following is typical were carried out in order to determine a suitable pH for the reaction. Benzamide digests were prepared with phosphate buffers of pH ranging from 5·00 to 8·04, the concentration of the amide being 0·210 g./100 ml. To 50 ml. of each were added 10 ml. of liver extract; the usual controls were also prepared. For titrating samples of the digest at pH 5·9 and 6·4 it was convenient to add 2 ml. of 0·1 N-NaOH before titration, while to samples of the digests at pH 6·9 and 7·4, 1 ml. 0·1 N-NaOH was added. Water (1 ml.) was added to the digest at pH 8·04. The results are shown in Fig. 2. It is evident that the pH (7·4) used throughout the main investigations is a suitable one.

Concentration of substrate. These experiments were carried out in order to compare the hydrolysis of benzamide and *p*-nitrobenzamide more closely, as in the main experiments the solutions of the latter were less than half the molarity of those of benzamide. In a typical experiment two digests were prepared, consisting of the buffer (pH 7·4) containing 0·21 g./100 ml. and 0·105 g./100 ml. benzamide respectively, together with the usual controls. The results of two experiments are shown in Fig. 3. Thus the percentage hydrolysis achieved is about 10 % greater with the more dilute substrate.

Effect of dialysis. The liver extract was prepared by the method described and its activity towards benzamide and *p*-nitrobenzamide determined. The remainder of the extract was dialyzed against running tap water for 17 hr. and its activity then determined, allowance being made for the dilution of the extract during dialysis.

The results of two experiments given in Fig. 4 show that there is no great difference in the equili-

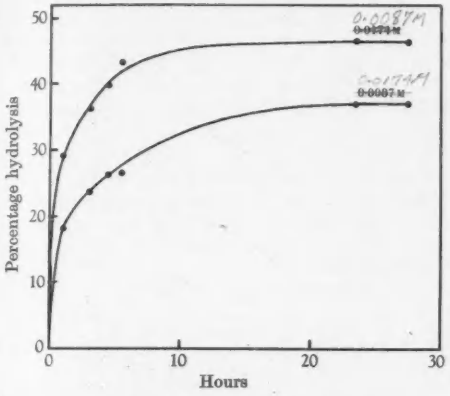

Fig. 3. Effect of concentration of substrate upon hydrolysis of benzamide by rabbit-liver extract.

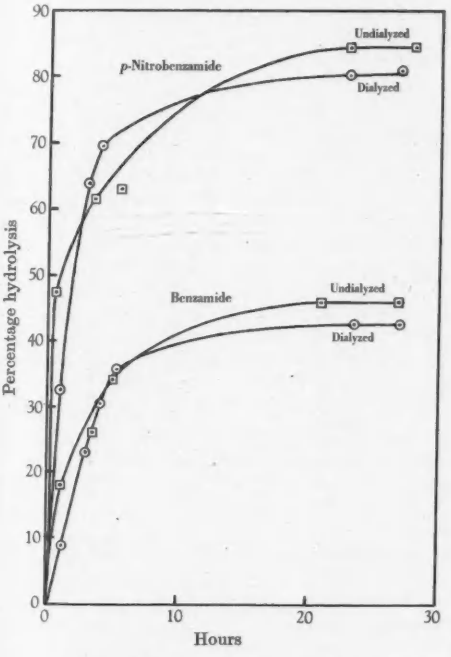

Fig. 4. Effect of dialysis upon activity of rabbit-liver extract.

brium values reached. In almost all experiments in the main section undialyzed extracts were used.

Hydrolysis of various amides by liver extracts. The hydrolytic effect of liver extracts was studied using the three isomeric hydroxy-, amino- and nitrobenzamides, *o*-bromobenzamide, benzamide, formamide, acetamide, propionamide, phenylacetamide, nicotinamide, asparagine and glutamine. The results of typical experiments are shown in Fig. 5, and those of all experiments are summarized in Table 3.

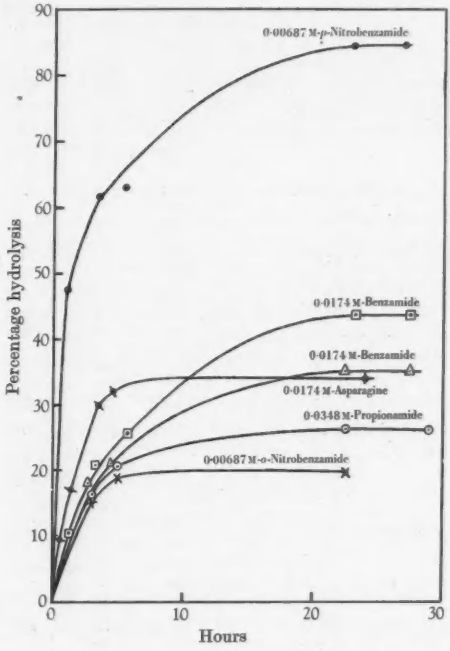

Fig. 5. Hydrolysis of amides by rabbit-liver extracts at pH 7·4.

Titration of ammonium salts. Solutions of the ammonium salts (25 ml., 0·0174 M) in buffer pH 7·4 were mixed with 5 ml. liver extract; 4 ml. samples of the mixture were treated with neutralized formaldehyde (0·5 ml. 40 %) and 0·1 N-NaOH (1 ml.) and centrifuged. Of the supernatant liquid 5 ml. were titrated against 0·02 N-NaOH. Blank determinations on the corresponding amides were carried out by adding liver extract (5 ml.) to 25 ml. of 0·0174 M-solutions of the amide in buffer pH 7·4; 4 ml. samples were withdrawn, treated with formaldehyde and titrated as above. The difference between the two titrations (corrected for volume) gives the titration due to the ammonia of the ammonium salt, and this represents the ammonia which would be formed by complete hydrolysis of the amide. The

results shown in Table 4 justify the use of the theoretical value as the basis for the calculation of percentage hydrolysis. Incubation of the mixture of ammonium salt and liver extract at 37° for 18 hr. did not affect the titres.

Table 3. *Hydrolysis of amides by rabbit-liver extracts*

Amide	Hydrolysis: In 10 hr. (%)	Hydrolysis: At equilibrium (%)	Average hydrolysis at equilibrium (%)
Benzamide (average values)	24	45	45
o-Hydroxybenzamide	0	0	0
	0	0	
	0	0	
	0	0	
m-Hydroxybenzamide	0	0	1
	0	0	
	2	4	
p-Hydroxybenzamide	0	0	6
	0	0	
	3	10	
	7	13	
o-Aminobenzamide	0	0	9
	0	0	
	3	7	
	8	15	
	16	32	
m-Aminobenzamide	7	13	7
	0	4	
	0	5	
	0	0	
	3	13	
p-Aminobenzamide	2	8	13
	3	11	
	0	0	
	0	26	
	11	20	
o-Nitrobenzamide	12	27	23
	13	28	
	15	15	
m-Nitrobenzamide	12	36	31
	22	45	
	3	17	
	24	24	

Table 3 (*cont.*)

Amide	Hydrolysis: In 10 hr. (%)	Hydrolysis: At equilibrium (%)	Average hydrolysis at equilibrium (%)
p-Nitrobenzamide	93	100	82
	68	79	
	43	91	
	96	96	
	42	60	
	38	50	
	100	100	
	86	86	
	64	64	
	84	84	
	84	84	
	50	70	
	100	100	
o-Bromobenzamide	8	14	21
	0	28	
	5	22	
Formamide	0	0	0
	0	0	
Acetamide	3	6	2
	0	3	
	0	0	
Propionamide	24	32	25
	5	10	
	9	26	
	6	30	
Phenylacetamide	7	19	10
	6	7	
	0	0	
	8	15	
	4	8	
Nicotinamide	9	9	13
	8	8	
	8	26	
	9	9	
Asparagine	30	37	32
	29	29	
	30	32	
Glutamine	16	16	22
	28	28	

Table 4. *Formol titration of* 0·0174 M*-solutions of ammonium salts, expressed as ml.* 0·02 N-NaOH

	Titration	Difference	Difference corrected for volume*	Calculated difference
Ammonium benzoate	6·75	—	—	—
Benzamide blank	4·27	2·48	2·73	2·9
Ammonium *p*-nitrobenzoate†	5·5	—	—	—
p-Nitrobenzamide blank	4·4	1·1	1·21	1·14
Ammonium *p*-hydroxybenzoate	7·07	—	—	—
p-Hydroxybenzamide blank	4·75	2·32	2·55	2·9
Ammonium *p*-aminobenzoate	7·1	—	—	—
p-Aminobenzamide blank	4·2	2·88	3·17	2·9

* I.e. titration due to ammonium salt.
† 0·00687 M-solution used owing to small solubility of amide.

Experiments with kidney extract

The scope of these investigations was limited by the small amounts of tissue available. In general it may be said that kidney extracts possess feeble amide-hydrolyzing properties. The rate of hydrolysis in some cases was found to be very slow, e.g. in one experiment *p*-nitrobenzamide was hydrolyzed to an extent of 35 % in 30 hr. while, in another, 60 hr. was required to bring about the same change. It is, perhaps, significant that Waelsch & Busztin (1937) found that aqueous extracts of horse kidney showed no enzymic activity of this type, but that glycerol extracts were able both to hydrolyze and synthesize benzamide. We found that glycerol extracts of rabbit kidney were not more active than aqueous extracts.

Experiments with proteolytic enzymes of animal origin

Trypsin. Solutions of benzamide (0·21 g./100 ml.); *p*-hydroxybenzamide (0·23 g./100 ml.); phenylacetamide (0·23 g./100 ml.); and *p*-nitrobenzamide (0·115 g./100 ml.) in phosphate buffer pH 8·04 were prepared and to each 3 % pancreatin (British Drug Houses Ltd.) (10–50 ml.) was added. Similar solutions treated with boiled enzyme solution served as controls. A further control consisted of buffer and enzyme solution without substrate. The solutions were incubated at 37° and samples (4 ml.) withdrawn at intervals and titrated as before. In no case was any significant hydrolysis observed.

Pepsin. Solutions of benzamide, *p*-hydroxybenzamide, *p*-nitrobenzamide and phenylacetamide were prepared as in the last experiment, but using an acetate buffer pH 1·2. To 50 ml. of each solution was added 3 % pepsin (British Drug Houses Ltd.) solution (10 ml.). These were incubated along with controls as above and samples withdrawn and titrated as before. No significant hydrolysis was observed. Similar experiments at pH 5·9, using a phosphate buffer, also gave negative results.

Inhibition of enzyme action by various substances

The discrepancy observed between the complete hydrolysis of phenylacetamide which occurs *in vivo* (Bray *et al.* 1946) and its relative stability to liver extracts *in vitro* suggested that phenylacetamide or a metabolite, e.g. phenylacetic acid or phenaceturic acid, might have an inhibiting effect upon the enzyme responsible for amide hydrolysis. Benzamide and *p*-nitrobenzamide were used as substrates. It was found that 30 mg. of these substances/60 ml. digest produced significant inhibition. Typical results are given in Table 5.

Table 5. *Inhibiting effect of various compounds upon hydrolysis of benzamide and p-nitrobenzamide by liver extracts*

Amide	Compound added	Hydrolysis at equilibrium (%)
Benzamide	—	61
	Phenaceturic acid	43
	Phenylacetic acid	34
	Phenylacetamide	30
p-Nitrobenzamide	—	85
	Phenaceturic acid	75
	Phenylacetic acid	57
	Phenylacetamide	46

It may be noted that phenaceturic acid, the chief metabolite of phenylacetamide in the rabbit, shows less inhibiting effect than either phenylacetamide or phenylacetic acid.

Hydrolysis of amides by acid and alkali

In order to compare the stability of the amides to acid and alkali with their stability to liver extracts, a series of experiments was carried out in which they were incubated with acid and alkali under standard conditions and the amount of ammonia formed estimated by aeration into standard acid. The conditions chosen in the case of acid were heating 0·001 mol. with N-HCl (25 ml.) in a boiling-water bath for 1 hr. Under these conditions benzamide was hydrolyzed to the extent of 33 %.

The alkaline conditions were similar except for using N-NaOH for hydrolysis, and resulted in 73 % hydrolysis of benzamide. Table 6 shows the results, together with those obtained from the enzyme experiments. The order of stability to acid and alkali is not greatly different, but stability to enzymic hydrolysis clearly depends upon other factors.

Table 6. *Hydrolysis of amides by liver extracts, acid and alkali*

Amide	Hydrolysis by liver extract at equilibrium (%)	Hydrolysis by N-HCl in 1 hr. (%)	Hydrolysis by N-NaOH in 1 hr. (%)
p-Nitrobenzamide	82	35	79
Benzamide	45	33	73
Asparagine	32	50	—
m-Nitrobenzamide	31	27	81
Propionamide	25	87	78
o-Nitrobenzamide	23	1	21
Glutamine	22	—	—
o-Bromobenzamide	21	—	—
Nicotinamide	13	28	73
p-Aminobenzamide	13	30	54
Phenylacetamide	10	87	79
o-Aminobenzamide	9	8	64
m-Aminobenzamide	7	26	70
p-Hydroxybenzamide	6	19	14
Acetamide	3	88	75
m-Hydroxybenzamide	1	32	55
o-Hydroxybenzamide	0	9	9
Formamide	0	83	81

The mechanism of amide hydrolysis by enzymes

When acid and alkali were used for the hydrolysis of amides the ammonia formed was estimated, whereas in the enzyme experiments the formol titration gave a measure of the acid formed. If the mechanism of the enzymic reaction is, like that catalyzed by acid and alkali, a hydrolytic one expressed

$$R.CONH_2 + H_2O \rightarrow R.COOH + NH_3,$$

then both methods of estimation should yield the same result. To confirm this, the progress of hydrolysis in some enzymic digests was followed by both methods. Fig. 6 shows that the two methods yield essentially the same results. The fact that no increase in acidity of the enzymic digests was apparent until formaldehyde was added suggests that the mechanism is a hydrolytic one.

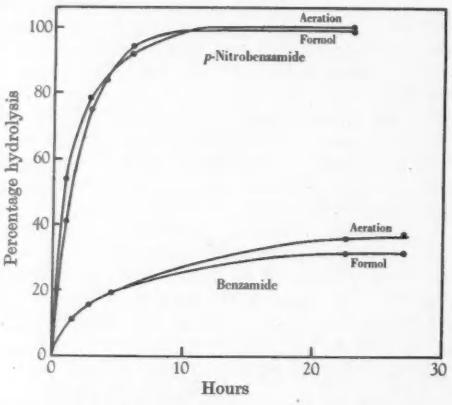

Fig. 6. Comparison of aeration and formol titration methods for estimation of hydrolysis of amides by rabbit liver extract.

The theoretical possibility of the enzymic conversion of the amide being due to an oxidative mechanism such as

$$R.CONH_2 + H_2O + O \rightarrow R.COOH + NH_2OH,$$

with subsequent conversion of the hydroxylamine to ammonia, has, however, been explored to the extent of testing for the presence of hydroxylamine. The Pucher and Day reaction (Pucher & Day, 1926) applied to various digests gave negative results up to 48 hr. incubation. The amounts of hydroxylamine which could be formed, however, are near the lower limit of sensitivity of the test. Hydroxylamine is converted in the tissues into nitrite and ammonia (Lewin, 1888–9):

$$2NH_2OH + O \rightarrow HNO_2 + H_2O + NH_3.$$

The same change is brought about by liver extracts. Amounts of hydroxylamine sufficient to give a good Pucher and Day test were added to liver extracts (140 mg./50 ml.) and incubated for 20 hr. The extracts then failed to give a Pucher and Day reaction, but the presence of nitrite was easily detected by the Griess-Ilosvay diazotization test. This test applied to the various amide digests was, like the Pucher and Day test, always negative.

Further evidence against an oxidative mechanism, at least an aerobic one, was provided by comparing the results obtained with digests incubated under the stated conditions with those from digests incubated in an atmosphere of oxygen or nitrogen. No significantly different results were obtained.

Other properties of liver extracts

The liver extracts used for the experiments described in this paper were capable of reducing the three isomeric nitrobenzamides to yield diazotizable material. They were also able to oxidize hydroxybenzoic acids and amides to dihydroxy compounds (cf. Bray *et al.* 1947), which could be detected by means of the colorations given with ferric chloride. Both these properties of the extracts are being investigated in more detail.

Attempts to obtain active dried liver preparations. When dried by means of acetone and ether, rabbit liver yielded inactive preparations. Dried ox liver, however, showed some enzymic activity. These investigations are being continued.

DISCUSSION

The main object of this research was to compare the hydrolysis of amides *in vivo* with that by enzymes *in vitro*. The results set forth above show that in general the activity of liver extracts in hydrolyzing amides is similar to that of the intact animal, since we have already shown that the metabolism of benzamide is virtually indistinguishable from that of benzoic acid (Bray *et al.* 1946), and that *in vivo* the carbamyl group of *p*-hydroxybenzamide is hydrolyzed to only a very slight extent (Bray *et al.* 1947). Investigations in progress in this laboratory suggest that *in vivo* *o*- and *m*-hydroxybenzamides are incompletely, if at all, hydrolyzed, that the three isomeric aminobenzamides are hydrolyzed only partially (Bray, Lake, Neale, Thorpe & Wood, 1948), and that *m*- and *p*-nitrobenzamides are probably completely hydrolyzed. Nencki (1873) states that acetamide is excreted unchanged by dogs, although his evidence does not preclude the possibility of a slight degree of hydrolysis.

So far, phenylacetamide appears to be the only exception to this general agreement, inasmuch as it

is completely hydrolyzed *in vivo** (Bray *et al.* 1946), but only to an extent of 10% by liver extracts. This apparent discrepancy may not be as great as it appears at first, since under the dynamic conditions prevailing in the living animal products of a reaction of this type are continuously removed, so that it can proceed further towards completion than under static conditions such as those used in *in vitro* experiments. Thus it is possible that amides which appear to be hydrolyzed with difficulty *in vitro* could be completely hydrolyzed *in vivo*. Phenylacetamide is excreted (as phenaceturic acid) much more slowly than benzamide or benzoic acid (as hippuric acid). Since, however, phenylacetic acid is eliminated as slowly as phenylacetamide, it would seem that it is the conjugation with glycine which is delayed rather than the hydrolysis of the amide.

The fact that the liver extracts used in this study were active in hydrolyzing asparagine and glutamine suggests that it might be profitable to investigate the possible identity of the liver enzyme(s) with asparaginase or glutaminase.

* These findings have been confirmed during the present investigation.

SUMMARY

1. The hydrolysis of 18 amides by rabbit-liver extracts has been studied and the results compared with some obtained on the rabbit *in vivo*.

2. A suitable pH for the reaction was 7·4.

3. Rabbit-kidney extracts behave in a qualitatively similar manner, but are less active.

4. The action of papain, pepsin and trypsin upon some of the amides is recorded.

5. There is considerable difference between the stability of the amides to hydrolysis by HCl or NaOH and by liver extracts.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The results for the percentage hydrolysis of amides by acid and alkali were obtained by Mr P. B. Wood, whom we wish to thank.

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Leaf Protease of Tobacco and other Plants

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The work described in this paper was undertaken to obtain information on the properties and activities of protein- and peptide-splitting enzymes of the green leaves of tobacco and other plants. These enzymes will be referred to as proteases.

Greenberg & Winnick (1945) list eleven plant proteases that have been obtained in a relatively high state of purity. All, with the exception of bromelin, pinguinain and solanain, are obtained from latex. These three are obtained from fruits, although bromelin also occurs in the green leaves of the pineapple. Other plant proteases which have been studied in detail are those of seeds, both dormant and germinating. Reports on proteases in the green leaves of plants that do not produce latex are few, and frequently amount to little more than an assertion of their presence, based in much of the early work on evidence that seems open to question.

The presence of proteases in the green leaf of tobacco has been noted by Loew (1900) (action on egg albumin; none detected on casein, fibrin or gelatin); Oosthuizen & Shedd (1913) (possibly present in cured leaves); Fodor & Reifenberg (1925, 1926) (present in green and dried leaves, peptone substrate); Silberschmidt (1934) (stem sap of *Nicotiana-Datura* grafts, peptone substrate); and by Nito & Kitamura (1936) ('trypsin' and 'erepsin' in fresh leaves, 'trypsin' lost on drying). Much of the diffi-

culty in working on the protease of green leaves is due to its low concentration—the activity/g. protein N of tobacco sap is seldom as high as 1% of that of a commercial papain preparation.

MATERIALS AND METHODS

Leaves of *Nicotiana tabacum* var. White Burley from glasshouse plants were used for most of the work, but leaves of other plants listed later were also used. The leaves were minced twice in a domestic meat mincer and the sap squeezed by hand through madapollam. The crude sap was freed from much cell debris and chloroplast material by centrifugation for 15 min. at 3000 r.p.m. (*c.* 1500 *g*); the supernatant fluid is subsequently referred to as 'sap'. The fibrous residue remaining after the expression of the crude sap was washed twice with a volume of distilled water approximately equal to that of the crude sap, and squeezed dry by hand. The residue is referred to as 'fibre'.

Measurement of protease activity

Changes in the viscosity of gelatin due to the action of protease were first used, but were abandoned as the low activity necessitated long periods of measurement during which spontaneous precipitation occurred leading to inaccuracy. The method finally adopted was that of measuring the increase of amino N produced by the incubation at 40° of protease-containing material with 0·8% (w/v) gelatin at pH 5. Michaelis buffer was used because it has a wide useful range and, unlike citrate, does not interfere with the subsequent estimation of amino N. Amino-acid N was determined by a modification of the method of Pope & Stevens (1939) which allows the use of small quantities of test material and eliminates interference by reducing materials.

Specificity of the method of Pope & Stevens (1939)

The method depends on the decomposition of copper phosphate at a controlled pH by amino-acids with the formation of amino-acid-copper complexes. Peptides also behave in the same way and their N is included in the amino N figures obtained. Since the method is here used for the estimation of extent of protein splitting, this lack of specificity is immaterial. Pope & Stevens found that about 80% of the amino N of completely hydrolyzed gelatin was estimated by this method. They used solutions containing from 0·028 to 0·4 mg. amino N/ml. after final dilution with buffered copper phosphate, and found that low results were obtained in the lower part of this range. In this work, final concentrations were only of the order of 0·004–0·024 mg. amino N/ml. and it was found that in this range about 80% of amino N was estimated when added as glycine which is all estimated at higher concentrations. Nevertheless the method is useful at these low concentrations and gives reproducible results.

A number of substances present in plant sap and fibre may be estimated as amino N, but would not interfere in the measurement of protease activity if their concentration remained unchanged during incubation, e.g. sugars, pectin and pectic acid, and organic acids. These are considered below.

Sugars. Sucrose and reducing sugars may account for up to 10% of the leaf dry weight and non-cellulosic polysaccharides (mainly starch) for a further 20–30% (Ward, 1942). Glucose and sucrose were found not to interfere in the concentrations likely to be encountered; further confirmation was obtained by adding salivary amylase to sap containing starch and soluble sugars, and incubating in the usual way with gelatin. At the end of incubation the amylase was still active in degrading added starch, but had produced no apparent increase in the protease activity measured.

Pectic acid. Methanol is present in tobacco sap (Holden, 1945) and its concentration (0·05 mg./ml.) indicates the possibility of the presence of about 0·5 mg./ml. of pectic acid. It was thought that the many free carboxyl groups of pectic acid might interfere. Its formation during incubation would be likely to interfere only with sap protease estimations, since when formed in fibre it appears to remain attached (Holden, 1945). It was found that pectic acid at a conc. of 1 mg./ml. did not increase the blank values.

Organic acids. Of the organic acids present in the leaf that are likely to interfere, oxalic may be excluded since it is not soluble at the pH values used (Vickery & Pucher, 1931). Malic acid does not interfere at the concentrations likely to be encountered, but citric acid does. Since malic acid may be converted to citric acid in water-cultured leaves in the dark (Vickery, Pucher, Wakeman & Leavenworth, 1937) interference from this cause must be considered. Most of the protease estimations were carried out in the presence of a reducing agent which lessens the likelihood of the change from malic to citric acid. It was found that the addition of 1 mg. of malic acid/ml. of sap did not affect the protease value found, and that sap dialyzed against 0·02 M-thioglycollic acid at 5° had lost no activity in spite of the loss of 75% of its initial 'amino N' content, and hence presumably of a major portion of its organic acids.

Amino-acid oxidase. If this were present low results would be obtained owing to destruction of amino-acids. Estimations of ammonia were made on samples incubated in the usual way and for twice the usual time and the difference between initial and final values was no more than 0·01 mg./ml.

Method of estimation

The enzyme solution (0·5 ml.) was pipetted into a 15 ml. tube and 0·4 ml. of distilled water added, followed by 0·1 ml. of a M-solution of activator or inhibitor. A 1% (w/v) gelatin solution (4 ml.) in Michaelis buffer (pH 5·0) at about 30° was then added and the contents of the tube mixed. A 2 ml. sample was then withdrawn into a small beaker, two drops of starch indicator added followed by iodine until the development of a permanent blue colour. The iodine stops the action of the enzyme and removes any reducing substances that would interfere in the subsequent titrations. After 30 min. excess iodine was removed quantitatively with 0·005 N-thiosulphate. From this point the procedure of Pope & Stevens (1939) was followed except that 15 ml. of copper phosphate suspension were used and the samples brought to a final volume of 25 ml. before filtration. Titration was carried out using 0·005 N-thiosulphate and a 1 ml. burette delivering below the surface of the fluid; 5 ml. samples of the filtrate were used with the addition of 0·25 ml. of glacial acetic acid and 0·5 ml. of

saturated KI. With starch indicator, the end point was not very sharp and increased accuracy could be obtained by the electrometric titration of Foulk & Bawden (1926) as modified by Bonner (1946). Gelatin and water blanks were run with each set of estimations, and for absolute values determinations were made on glycine solutions.

Immediately after the initial samples had been taken a drop of chloroform was added to each tube, the tubes stoppered and incubated at 40° for 24 hr. At the end of this time, a second 2 ml. sample was taken and treated in the same manner as the first. From the difference in the values obtained by titration of initial and final samples the number of units/ml. was calculated. If the activity of fibre is to be estimated 1 g. is weighed into the tube and double amounts of water, activators or inhibitors and gelatin solution are added. Samples of 2 ml. are taken and treated like the samples from estimations in liquids.

The unit of activity is defined as that activity which leads to the production of 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 for 24 hr. at 40°. The protease unit is the amount of protease which produces 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 with 0·8 % (w/v) gelatin for 24 hr. at 40°.

RESULTS

Preliminary observations on the protease

Protease activity is shown both by sap and fibre of tobacco and other leaves, about 60 % of the total activity of healthy tobacco leaves being present in the sap. The activity of both sap and fibre was increased by reducing agents such as sodium thioglycollate, the reagent most frequently used in routine experiments. Activators and inhibitors were used at a final concentration of 0·02 M. Sodium sulphide, sodium hyposulphite, potassium cyanide, ascorbic acid, cysteine and glutathione were also effective as activators. Activity was unaffected by manganese, was reduced to about 25 % of its thioglycollate value by iodoacetic acid and was abolished by copper. The pH optimum of the protease was found to be *c.* 5 with gelatin as substrate. The enzyme has thus many properties in common with other plant enzymes of the papain type.

Method of partial purification

If tobacco sap is heated to 55° for a few minutes much of the normal plant protein is denatured and can be removed by centrifugation (Bawden & Pirie, 1938). Such heating leads to some loss of total activity, but may increase the purity (i.e. activity/g. protein N) by as much as tenfold. The addition of one twentieth volume of ethanol to heated sap adjusted to pH 4·2 gives a further increase in purity. Table 1 shows the progress of a typical purification. The purification at each step may vary widely, but the overall results are usually similar to the example given. Precipitation of the enzyme has been attempted under many conditions, but does not seem to give as satisfactory results as methods based on the precipitation of impurities. Attempts have been made to prepare fractions of sap protein with enhanced protease content by using $(NH_4)_2SO_4$, ethanol and acetone. These have not been successful, all the fractions prepared having much the same activity. Protease present on fibre is not removed by water, 5 % NaCl or glycerol solutions and has not been purified.

Properties of the enzyme

Fig. 1 shows the variation of the activity of the protease with the pH of the medium. Determinations were made by adding buffered 1 % gelatin to samples of sap, adding activator and water and determining the pH after the initial and final samples were taken. Michaelis buffer was used.

Variation of activity with substrate concentration was measured in the usual way. Difficulty was encountered in handling samples containing more

Table 1. *Partial purification of tobacco-sap protease*

	Protease (units/ml.)	Protein N (mg./ml.)	Protease (units/g. protein N)	Total protease (units)	Protease units as % of initial value
(*A*) Crude sap	0·39	2·22	176	35·1	100
(*B*) *A* spun*	0·40	1·82	247	34·8	99
(*C*) *B* heated and spun*	0·28	0·82	342	21·8	62
(*D*) *C* spun at pH 4·2*	0·25	0·34	735	18·2	52
(*E*) *D* + 5 % (w/v) ethanol, spun*	0·21	0·23	910	15·3	44
(*F*) *E* concentrated by ultrafiltration and dialyzed	—	—	—	—	—
(*G*) Fraction of *F* insoluble in Michaelis buffer, pH 5	0·09	0·175	515	2·1	6
(*H*) Fraction of *F* soluble in Michaelis buffer, pH 5	0·33	0·11	3000	7·65	22

* Refers only to the supernatant liquid.

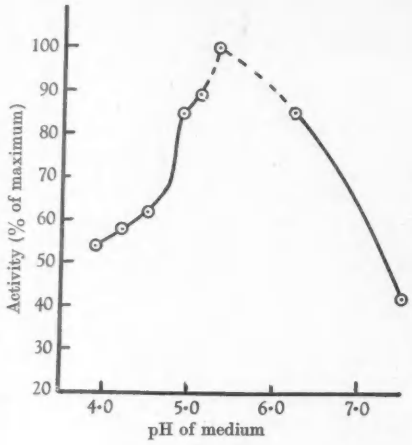

Fig. 1.

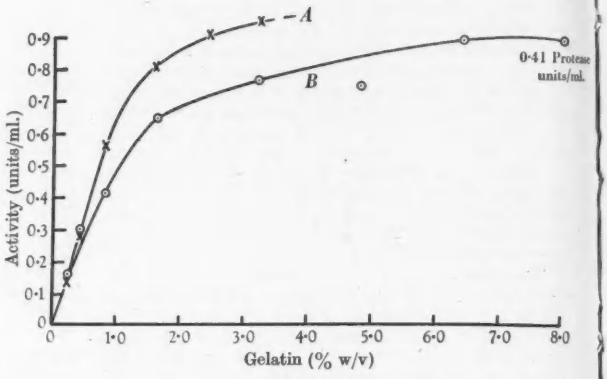

Fig. 2.

Fig. 1. Protease activity at various pH values expressed as percentage of the maximum activity found. Portions of the same sample of partially purified tobacco sap were used for all determinations.

Fig. 2. Variation of activity with substrate concentration in partially purified samples of tobacco-sap protease containing (*A*) 0·56 and (*B*) 0·41 protease units/ml.

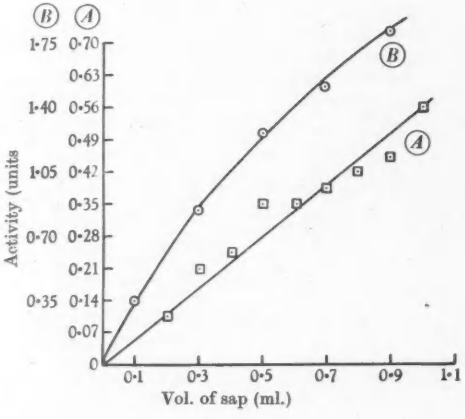

Fig. 3.

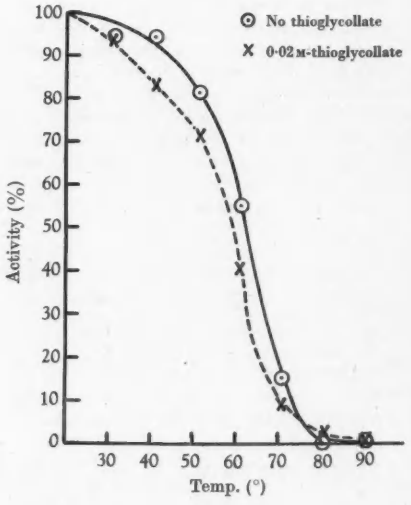

Fig. 4.

Fig. 3. Variation of activity with protease concentration, (*A*) 0·28 protease unit/ml., (*B*) 1·22 protease units/ml.

Fig. 4. The effect of heat on tobacco-sap protease. Portions of partially purified protease were heated for 5 min. at the temperatures indicated, in the absence of added substrate. Activities subsequently measured are expressed as percentages of that of the unheated sap.

than 4% (w/v) gelatin owing to gelling during sampling. Typical results are shown in Fig. 2. The concentration of gelatin which gives half the maximum velocity of hydrolysis is about 0·8%.

An approximately linear relationship was found between the amount of enzyme present and the extent of hydrolysis over the enzyme concentration range usually employed (Fig. 3). The effect of heat

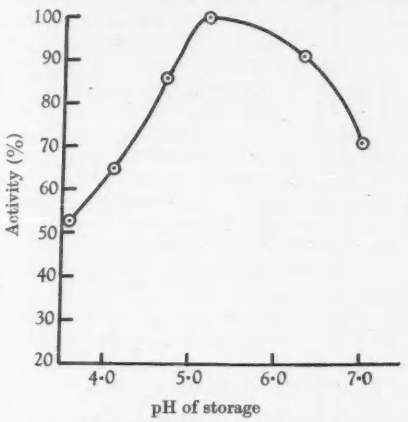

Fig. 5. The effect of pH on the stability of tomato-sap protease kept at 5° for 20 hr. The activities are expressed as percentages of the maximum observed activity in this experiment.

on enzyme activity was determined by heating samples of sap at pH 5 in the absence of added substrate to a desired temperature, maintaining it at that temperature for 5 min. and cooling rapidly. The activity of the heated samples was then measured in the usual manner. Fig. 4 shows that some inactivation occurs even at 40° and that the presence of reducing agent has little effect. The influence of the temperature of incubation on the activity measured was measured at four temperatures. Enzyme action at 25° was 57%; at 20°, 25%; at 5°, 14% of the activity at 40° using gelatin as substrate. The pH of maximum stability on storage at 5° was measured on samples of tomato sap. Values of pH were measured 1 hr. after adjustment and at the end of 20 hr. at 5° and were found not to have changed, except for the sample initially at 6·5 which had changed to 6·3 and the sample at 7·4 which had changed to 7·0. Final pH values have been used in Fig. 5. The pH of maximum stability seems to be in the region of the optimum for protease activity, about pH 5.

The course of protease activity in the presence of added substrate was followed for both sap and fibre for 7 days at 40°. In Fig. 6 the values for the sap have been multiplied by 3·3 in order to show an equivalent activity.

A number of substrates have been tested with tobacco-leaf protease. The protein present in both crude and spun sap is split to some extent, but the amount of amino N released is small compared with that which occurs in the presence of added gelatin.

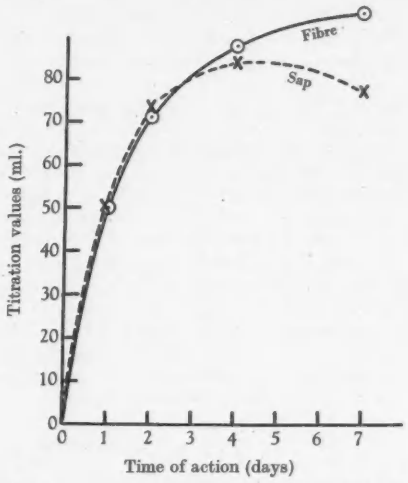

Fig. 6. Progress curve for the hydrolysis of gelatin (0·8%, w/v) by tobacco protease over an extended period. Titration values for equal samples withdrawn at intervals are plotted against time. The values for sap have been multiplied by 10/3.

Table 2. *Activity of tobacco protease with various substrates*

	(units/ml.)
Crude sap*	0·18
Spun sap†	0·03
Spun sap + gelatin	0·45
Spun sap + wheat gluten	0·40
Spun sap + egg albumin	0·07
Spun sap + peptone	0·45
	(units/g. dry wt.)
Washed fibre	3·10
Washed fibre + gelatin	4·50

* Final concentration of sap protein *c.* 0·14%.
† Final concentration of sap protein *c.* 0·11%.

Table 2 shows the amount of amino N formed by tobacco protease acting on various substrates and its own proteins at pH 5. Added substrates had a final concentration of 0·8% (w/v); with sap alone the usual volume of 0·5 ml. was used.

Inhibition by iodoacetate

Tobacco-leaf protease is not completely inhibited in the presence of 0·02M or even 0·1M-iodoacetate though no activity can be detected in the presence of 0·02M-copper salts. It is possible that the pro-

tease is a mixture of enzymes, some stable to iodoacetate and some inhibited by it. The ratio of full activity in the presence of thioglycollate to the residual activity shown in the presence of iodoacetate does not change appreciably during the course of preliminary purification, and attempts to demonstrate the existence of different pH optima in the presence of thioglycollate and the presence of iodoacetate have not been successful.

Leaf protease of other plants

The leaves of a number of plants have been examined for the presence of protease. In all those so far examined, the enzyme is activated by thioglycollate and is active at pH 5. Table 3 lists some of the plants tested. In those for which a range of values is given, samples varying in fertilizer treatment were used. The leaves of some plants do not yield sap on mincing (hollyhock, *Althea rosea*; box, *Buxus sempervirens*), while laurel (*Prunus laurocerasus*) gives a sap containing only traces of protein. These leaves were however found to contain protease in amounts similar to those in tobacco.

protease content may be found in plants grown under different cultural conditions (Table 3: tobacco, potato, beet), and that caution must therefore be exercised in drawing conclusions from the examination of plants of unknown antecedents and cultural background. In spite of these variations in protease concentration, none of the plants examined has approached the protease content of the pineapple, which is typical of those plants containing the well characterized plant proteases. The plants examined do, however, appear to form a homogeneous group when protease contents are compared on a basis of units/g. protein N, or units/g. total N. All the values quoted for sap protease/g. protein N (with the exception of pineapple) fall within the range found in tobacco, although the concentrations in sap on a volume basis may differ by a factor of six.

It is probable that the proteases described in this paper have not been examined in any detail before because they occur in such small concentrations. It must be assumed, however, that the protease levels found are adequate for the metabolic needs of the plants; if the total plant protein were equivalent as substrate to gelatin, all the leaf protein could be

Table 3. *Sap and fibre protease content of a number of plants*

		Sap (units/ml.)	Sap (units/g. protein N)	Fibre (units/g. dry wt.)	Fibre (units/g. N)
SOLANACEAE:					
Tobacco, *Nicotiana tabacum*	(1)	0·03–0·63	25–1040	0·8–12·4	20–270
Potato, *Solanum tuberosum*	(2)	0·60–2·18	150–780	8·6–13·8	160–390
Woody nightshade, *Solanum dulcamara*		1·02	1020	—	—
Tomato, *Lycopersicum esculentum*		0·79	370	—	—
CHENOPODIACEAE:					
Sugar beet, *Beta vulgaris*	(3)	0·14–0·67	60–400	1·8–6·7	70–320
CUCURBITACEAE:					
Marrow, *Cucurbita pepo* var. *ovifera*		0·42	244	1·2	51
Bryony, *Bryonia dioica*		0·21	130	1·6	31
CRUCIFERAE:					
Cabbage, *Brassica oleracea*		0·35	263	12·5	330
LEGUMINOSAE:					
Bean, *Phaseolus vulgaris*		0·63	375	1·02	133
GRAMINEAE:					
Wheat, *Triticum vulgare*	(4)	2·7–4·1	350–580	2·5–3·5	120–210
Cocksfoot, *Dactylis glomerata*		0·14	202	0·63	77
BROMELIACEAE:					
Pineapple, green leaves of *Ananas sativus*		6·5	13,000	8·85	—
Papain (British Drug Houses, 0·005 mg. N/ml.)		0·42	84,000	—	—

Results are from single determinations except (1)–(4) which are the ranges with (1) 80 samples, (2) 16 samples, (3) 54 samples, (4) 6 samples of plants with varied fertilizer treatments at different seasons.

DISCUSSION

The methods described in this paper have been used to measure the protease content of plants grown under different fertilizer conditions and infected with different viruses. It is hoped to publish these results shortly. They show that very wide variations in

split by its enzymes in 2–10 days at pH 5 and 40°. Thus it appears that the levels in some plants are abnormally high. It is difficult to see what evolutionary advantage the remarkably high protease activity in latex and fruits has for the plants concerned, since an abnormal resistance to pathogens does not seem to be conferred; pineapple, papaw and fig all suffer from virus diseases.

No evidence has been found to show that the protease is a mixture of enzymes or that the protease of one plant is distinguishable from another. It has been customary to name a protease after the generic name of the plant in which it is found. This custom, both here and in future work, implies an indefinite extension in the number of proteases. It is therefore recommended that the proteases described in this paper shall be considered identical until evidence to the contrary is adduced, and that they be called leaf proteases, adding where necessary the name of the plant.

SUMMARY

1. Protease activity is shown by both sap and fibre of the green leaves of the plants examined.

2. This activity is less than one tenth of that shown by the leaves of pineapple.

3. The activation of the enzyme by reducing agents and the pH optimum suggest that the protease is similar to the papain enzymes.

4. It is suggested that the enzyme be referred to as 'leaf protease' until evidence is obtained of the existence of more than one protease in the leaf or of differences between the leaf proteases of different plants.

5. The method of Pope & Stevens (1939) has been modified to allow smaller amounts of amino nitrogen to be estimated in the presence of reducing agents.

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The Free Amino Groups of Haemoglobins

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Since the original work of Reichert & Brown (1909) on the crystal form of the haemoglobins from a large number of species, many workers have shown them to differ in chemical and physical properties. Among the properties studied were the CO-combining capacity (Douglas, Haldane & Haldane, 1912), oxygen dissociation (Macela & Seliškar, 1925), absorption spectra (Anson, Barcroft, Mirsky & Oinuma, 1925), amino-acid composition (Vickery & White, 1933; Block, 1934), rate of alkali denaturation (von Krüger, 1887) and electrophoretic mobility (Landsteiner, Longsworth & van der Scheer, 1938). Apart from the occasional ability of haemoglobins of closely related species such as the horse and the donkey to form mixed crystals, the property which has brought out marked similarities as well as differences is their immunological behaviour. The most extensive survey was made by Hetkoen & Boor (1931), who showed that, although the haemoglobins were species-specific, cross reactions occurred between the antibodies and antigens of allied species. The extensive work of Landsteiner (1945) and others has suggested a relationship between the antigenic behaviour of a protein and its chemical structure. As an investigation of the free α-amino-groups of a protein brings out some details of the molecular structure (Sanger, 1945), it was of interest to make a comparative study of the terminal residues of several haemoglobins, and to see if any correlation between structure and immunological behaviour could be detected.

EXPERIMENTAL

Preparation of DNP-amino-acids

The method for the preparation of 2:4-dinitrophenylamino-acids (DNP-amino-acids) using 1:2:4-fluorodinitrobenzene (FDNB) has been previously described (Sanger, 1945).

Details for the purification of the various derivatives are given below.

N-2:4-*Dinitrophenyl*-DL-*isoleucine*. The amorphous product obtained by acidifying the alkaline reaction mixture was filtered off and crystallized from methanol-water; m.p. 166°. (Found: C, 48·1; H, 4·9%. $C_{12}H_{15}O_6N_3$ requires C, 48·6; H, 5·0%.)

N-2:4-*Dinitrophenyl*-DL-*methionine*. On acidification of the reaction mixture an oil separated which was extracted into ether. The ether extract was dried over anhydrous Na_2SO_4 and taken to dryness leaving an oil which crystallized overnight. Yield 80% theoretical. The material was recrystallized by dissolving in dry ether and adding an equal volume of light petroleum (b.p. 80–100°). On evaporation of the ether crystalline plates were obtained, m.p. 117°. (Found: C, 41·9; H, 4·2; N, 13·3%. $C_{11}H_{13}O_6N_3S$ requires C, 41·9; H, 4·1; N, 13·3%.)

N-2:4-*Dinitrophenyl*-L-*proline*. On acidification an oil was formed which solidified overnight. Yield 85% theoretical. After recrystallization from ether-light petroleum and acetic acid-water it had m.p. 137°. (Found: C, 46·8; H, 3·9; N, 14·6%. $C_{11}H_{11}O_6N_3$ requires C, 47·0; H, 3·9; N, 14·9%.)

N-2:4-*Dinitrophenyl*-L-*tryptophan*. A gum which was obtained on acidification crystallized readily from aqueous methanol; m.p. 175° (decomp.). (Found: C, 55·5; H, 3·7%. $C_{17}H_{15}O_6N_4$ requires C, 55·0; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*lysine*. The amorphous precipitate obtained on acidification was crystallized from formic acid with some difficulty and was recrystallized from methanol-water; m.p. 146°. (Found: C, 45·2; H, 3·73; N, 17·8%. $C_{18}H_{18}O_{10}N_6$ requires C, 45·1; H, 3·77; N, 17·6%.)

N-2:4-*Dinitrophenyl*-DL-*aspartic acid*. This compound crystallized on acidification and was recrystallized from water; m.p. 196° (decomp.). (Found: C, 40·2; H, 3·0; N, 13·9%. $C_{10}H_9O_8N_3$ requires C, 40·2; H, 3·0; N, 14·0%.)

N-2:4-*Dinitrophenyl*-DL-*serine*. The oil which separated on acidification crystallized on standing and was recrystallized from methanol; m.p. 199°. (Found: C, 39·5; H, 3·2; N, 15·4%. $C_9H_9O_7N_3$ requires C, 39·8; H, 3·3; N, 15·5%.)

N-2:4-*Dinitrophenyl*-DL-*threonine*. Crystallized on acidification and recrystallized from aqueous methanol; m.p. 152°. (Found: C, 41·7; H, 3·8%. $C_{10}H_{11}O_7N_3$ requires C, 42·1; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*cystine*. On acidification an amorphous precipitate separated. It was crystallized from ethylene glycol monoethyl ether-water and recrystallized from acetic acid-water. Yield 80% theoretical; m.p. 109°. (Found: C, 38·2; H, 3·1; S, 11·2%. $C_{18}H_{16}O_{12}N_6S_2$ requires C, 37·8; H, 2·8; S, 11·2%.)

N^1-2:4-*Dinitrophenyl*-L-*arginine*. After removal of the ethanol from the reaction mixture, the residue was treated with water. *DNP-arginine* was insoluble and was filtered off and washed with ethanol and ether. It was recrystallized by solution in dilute HCl and neutralization with ammonia; yield 70%, m.p. 252° (decomp.). For analysis it was crystallized from conc. HCl as the hydrochloride; m.p. 168°. (Found: C, 37·7; H, 4·4; N, 22·5%. $C_{12}H_{16}O_6N_6$.HCl requires C, 38·3; H, 4·5; N, 22·6%.)

The DNP-derivatives of glutamic acid and hydroxyproline have not yet been obtained in crystalline form.

N^5-2:4-*Dinitrophenyl*-L-*lysine*. A simpler preparation of this compound similar to that described for DNP-ornithine (Sanger, 1946) has now been achieved. L-Lysine (0·5 g.) was dissolved in 10 ml. water and $CuCO_3$ added slowly to the boiling solution. The excess $CuCO_3$ was filtered from the dark blue solution and washed with 2–3 ml. water. Excess $NaHCO_3$ was added followed by a solution of 1·5 g. DNFB in 20 ml. ethanol and the mixture shaken for 2 hr. at room temperature. The yellow-green precipitate was filtered off and washed with water, ethanol and ether. It was then suspended in 5 ml. water and sufficient N-HCl added to produce a clear solution. This solution was cooled in ice, H_2S bubbled through for 2 min., a trace of charcoal added and the mixture filtered immediately. The filtrate was rapidly taken to dryness *in vacuo*, crystallized from water and recrystallized from 20% HCl; yield 0·45 g.

Chromatographic separation of DNP-derivatives

The methods of separation and identification of the DNP-derivatives are essentially the same as those previously described (Sanger, 1945). It should be emphasized again that the R_F values recorded in that paper apply only to a certain batch of silica gel (cf. Tristram, 1946). The absolute, and to a lesser extent the relative, R_F values vary considerably with different batches of gel. It appears that the DNP-amino-acids are partly held in the aqueous phase by adsorption on the silica and that the fractionation is not entirely the result of liquid-liquid partition. In general an adsorbent gel, giving low R_F values, produces the maximum resolution for any given solvent, though increased 'tailing' of the bands sometimes prevents clean separation. Table 1 shows a scheme of fractionation which has been found applicable to most batches of gel used in this laboratory.

Stability of DNP-amino-acids to acid hydrolysis

*Hydrolysis in boiling 5·7*N-HCl. In order to ascertain whether any terminal amino-acids might be overlooked due to the instability of their DNP-derivatives during hydrolysis, a survey was made of the rates of breakdown of all the DNP-derivatives.

A standard solution was made of DNP-amino-acid in 5·7N-HCl. A suitable portion was taken, some globin was added in case the rate of breakdown was influenced by the presence of hydrolytic products of protein, and the mixture boiled under reflux for the required time. The hydrolysate was extracted as previously described and the extract passed through appropriate columns in order to separate any breakdown products. The DNP-amino-acid was collected and the breakdown estimated by colorimetric comparison with the original solution. The results obtained are shown in Table 2.

It is clear that, with the exception of proline (Consden, Gordon, Martin & Synge, 1946), all amino-acids with a free amino or imino group could be detected using this method of hydrolysis. It was found, however, that during hydrolysis, some DNP-amino-acids gave rise to coloured breakdown products which were liable to cause confusion in the chromatographic separation. DNP-methionine and DNP-tyrosine both produced a coloured 'artefact' band stationary on a chloroform column. DNP-cystine yields a decomposition product which hardly moves on a 3% butanol-chloroform column. DNP-tryptophan on hydrolysis gave

Table 1. *Scheme of fractionation of ether-soluble DNP-amino-acids*

(Numbering refers to fractions and subfractions.)

a coloured product which remained in the aqueous solution during ether extraction and moved fast on a 66% -methyl ethyl ketone-ether column. Their behaviour on other columns distinguishes these breakdown products from the DNP-amino-acids. No other DNP-amino-acids give rise to 'artefact' bands with R_F values less than 1·0 on a chloroform column, though some of them give a band moving fast which is probably 2:4-dinitroaniline.

Table 2. *Approximate breakdown of DNP-amino-acids on acid hydrolysis*

Amino-acid derivative	Hydrolysis in boiling 5·7 N-HCl: Time of hydrolysis (hr.)	Hydrolysis in boiling 5·7 N-HCl: Amount unchanged (%)	Hydrolysis for 16 hr. in 12 N-HCl at 105° (minimal figures). Amount unchanged (%)
DNP-alanine	12	80	75
DNP-arginine	12	90	75
DNP-aspartic acid	24	60	75
bis-DNP-cystine	12	25	0
DNP-glutamic acid	12	75	75
DNP-glycine	8	40	50
DNP-hydroxyproline	4	40	50
DNP-isoleucine	12	80	75
DNP-leucine	12	80	75
bis-DNP-lysine	8	95	75
ε-DNP-lysine	12	95	75
DNP-methionine	12	75	75
DNP-phenylalanine	12	70	50
DNP-proline	2	10	50
DNP-serine	12	90	75
DNP-threonine	24	90	75
DNP-tryptophan	12	90	0
DNP-tyrosine	12	75	50
DNP-valine	12	80	75

Hydrolysis in approx. 12 N-HCl *at* 100°. Many conditions of hydrolysis were investigated in order to find a method suitable for the estimation of DNP-proline. It was found that if DNP-proline and globin were heated in a sealed glass tube with 12 N-HCl at 105° for 16 hr., less than 50% decomposition was produced. The stability of DNP-hydroxyproline at the higher acidity was greater than in boiling 5·7 N-HCl, but with a number of other DNP-amino-acids (e.g. tryptophan and cystine) the decomposition was greatly increased. Earlier observations had suggested that proline might be a terminal amino-acid in salmine, and hence this protein was used to test the efficiency of the method of hydrolysis. Using 12 N-HCl in a sealed tube we were able to confirm that proline is in fact the only terminal amino-acid of salmine. This was expected from the inability of salmine to yield nitrogen in the Van Slyke apparatus. The great lability of DNP-proline had not been observed during the earlier experiments carried out on insulin (Sanger, 1945) and gramicidin S (Sanger, 1946). Using the above method of hydrolysis we have been able to confirm that in neither is proline a terminal residue.

Reaction of FDNB with valine

In order to test whether the reaction with valine is complete in 2 hr., two samples of DL-valine were allowed to react with FDNB for 2 and 16 hr. respectively, the DNP-valine being estimated after purification on a chloroform column. The results obtained are shown in Table 3.

Table 3. *Reaction of FDNB with valine*

Time of reaction (hr.)	Yield (mg. α-amino N)
2	0·22
16	0·24
Calculated	0·225

The discrepancy is close to the experimental error and is not considered significant. In order to ensure that the time necessary for the reaction was not increased when the amino-acid was in combination in a protein, the effect of increasing the time of reaction of FDNB with haemoglobin from 2 to 24 hr. was investigated. Again the quantitative results showed no significant difference.

Preparation of the DNP-globins

Horse haemoglobin was prepared by the method of Adair & Adair (1934). A similar method was used for goat and donkey haemoglobins, but crystallization was induced by dialysis against ammonium sulphate solution of suitable concentration. The cow, sheep and human haemoglobins were prepared by the method of Boor & Hetkoen (1930), human foetal haemoglobin from the blood of a 30-week foetus by the method of Haurowitz (1935), and horse myoglobin by the method of Theorell (1932).

The DNP-globins were prepared as described for DNP-insulin (Sanger, 1945), but two types of preparation were made. In the first the haem was removed (Anson & Mirsky, 1934) before the reaction with FDNB, and in the second afterwards. This was done in order to determine if the coupling of the haem with the globin blocked any of the free amino groups. The amount of globin in the air-dried DNP-globins was calculated from the amide content as previously described. The values are shown in Table 4.

Table 4. *Globin content of air-dried DNP-globins*

DNP-globin	Globin (%)
Cow	72
Donkey	75
Goat	75
Horse	76
Human adult	77
Human foetal	80
Sheep	70
Horse myoglobin	70

Measurement of the DNP-amino-acids

The DNP-amino-acids were measured colorimetrically with a photoelectric absorptiometer after solution in N-HCl or 1% (w/v) $NaHCO_3$. Contrary to a previous statement it was found that the colorimetric curves obtained with the DNP-derivatives of the L- and DL-amino-acids studied were identical. The losses of DNP-valine and DNP-methionine during hydrolysis in the presence of globin are shown in Table 5.

Table 5. *Breakdown of DNP-amino-acids on acid hydrolysis*

Method of hydrolysis	DNP-amino-acid	Time of hydrolysis (hr.)	Amount unchanged (%)
Boiling 5·7 N-HCl	DNP-valine	16	75
		24	64
	DNP-methionine	8	85
		24	46
12 N-HCl at 105°	DNP-valine	16	90
		40	75

As the factor which is used to correct for breakdown of DNP-amino-acids during hydrolysis is probably the principal source of error, the time of hydrolysis was always reduced until peptides were detected. In the globins about 10% of DNP-valine was still in peptide form after 16 hr. hydrolysis with 5·7 N-HCl whilst only a trace of a methionyl peptide was detected after 8 hr. hydrolysis. DNP-valyl peptides could just be detected after 40 hr. hydrolysis in 12 N-HCl at 100°. It is interesting to note that the intensity of the colour of DNP-valine and DNP-glycine is considerably reduced when they are in peptide form. Hence, peptides, if present, were separated on the columns, hydrolyzed further and estimated separately.

RESULTS

Identification of the terminal residues in haemoglobins

In human adult, human foetal, horse and donkey haemoglobins only one terminal amino-acid was found. By passing the DNP-derivative obtained from the hydrolysate through a number of different columns and comparing the R_F values with that of synthetic DNP-amino-acids, the terminal residue was found to be valine. This was confirmed by showing that the DNP-derivative could be separated on the appropriate column from all other DNP-amino-acids except DNP-valine. In a similar manner it was demonstrated that cow, sheep and goat haemoglobins contain two terminal residues which were identified as valine and methionine. Glycine was found to be the only terminal amino-acid in horse myoglobin.

Estimation of the terminal residues in haemoglobins

The results obtained are listed in Table 6. With the exception of the human foetal haemoglobin the figures for the numbers of the free amino groups

Table 6. *The numbers of free amino groups of haemoglobins*

Globin	Conditions of hydrolysis	No. of free α-amino groups/mol. of mol. wt. 64,000: Valine	Methionine	No. of free lysine ε-NH_2 groups/mol. wt. of 64,000
Horse	24 hr. boiling 5·7 N-HCl	6·3, 6·1, 5·8	Nil	42, 41, 40
	16 hr. boiling 5·7 N-HCl	5·9	—	—
	40 hr. 100° 12 N-HCl	6·0	—	—
	Mean	6·0	Nil	41
Donkey	16 hr. boiling 5·7 N-HCl	5·6, 6·0	Nil	40, 42
	Mean	5·8	Nil	41
Cow	24 hr. boiling 5·7 N-HCl	2·2, 2·0	1·9, 2·3	46, 48
	Mean	2·1	2·1	47
Goat	16 hr. boiling 5·7 N-HCl	2·4	1·8	47, 49
	8 hr. boiling 5·7 N-HCl	2·3, 2·0	1·7, 1·8	—
	Mean	2·3	1·8	48
Sheep	24 hr. boiling 5·7 N-HCl	2·1, 2·1, 2·0	2·0, 1·9, 1·9	46, 48, 48
	8 hr. boiling 5·7 N-HCl	2·1	2·2	—
	40 hr. 100° 12 N-HCl	2·3	—	—
	Mean	2·1	2·0	47
Human adult	24 hr. boiling 5·7 N-HCl	5·1, 4·8, 5·3	Nil	42, 43, 45
	16 hr. boiling 5·7 N-HCl	4·8	—	—
	40 hr. 100° 12 N-HCl	5·2	—	—
	Mean	5·1	—	43
Human foetal	24 hr. boiling 5·7 N-HCl	2·5, 2·6, 2·7	Nil	47, 45, 50
	Mean	2·6	—	47
		Glycine/mol. wt. 17,000		Lysine/mol. wt. 17,000
Horse myoglobin	4 hr. boiling 5·7 N-HCl	1·2, 1·2, 1·3		17, 21, 24
	2 hr. boiling 5·7 N-HCl	0·9, 1·1		20
	8 hr. 100° 12 N-HCl	0·9		—
	Mean	1·1		20

Table 7. *Summary of results*

Haemoglobin	Assumed mol. wt.	Terminal residues	Free lysine ε-amino groups
Horse	66,000	6 valine	41
Donkey	66,000	6 valine	41
Human adult	66,000	5 valine	43
Cow	66,000	2 valine, 2 methionine	47
Sheep	66,000	2 valine, 2 methionine	47
Goat	66,000	2 valine, 2 methionine	48
Human foetal	66,000	2·6 valine	47
Horse myoglobin	17,000	1 glycine	20

have been approximated to the nearest whole number and are summarized in Table 7.

No accurate figure is yet available for the molecular weight of human foetal haemoglobin. The sedimentation constant (Andersch, Wilson & Menten, 1944) suggests a molecular weight about half that of the adult haemoglobin, but as the diffusion constant was not measured the true value could not be calculated. The results obtained would suggest one terminal residue/molecule of mol.wt. 25,000 or two/molecule of mol.wt. 50,000.

The accuracy of the figures obtained is of particular importance in assessing the significance of the difference between the six terminal valyl residues found in horse haemoglobin and the five found in human haemoglobin. When a breakdown correction of 25% is used there must be doubt as to the importance of a difference of only 15–20%; however, the consistency of the figures obtained under a variety of conditions of hydrolysis and the absence of any overlapping justifies, it is felt, the conclusion that there is a structural difference between the two proteins.

DISCUSSION

The significance of the results will be discussed, first, as to the light they can throw on the molecular structure of the haemoglobins, and secondly, as to the correlation that can be found between the structural pattern and the immunological specificity of the molecule.

Structure. The number of free α-amino groups gives the number of open peptide chains present in the protein molecule. Possibly there also exist in the molecule cyclic chains or chains branching off the ω-carboxyl groups of glutamic or aspartic acid. Such chains would contain no free α-amino groups. The presence of cyclic chains has been demonstrated in gramicidin S (Sanger, 1946) and tyrocidine (Christensen, 1945) and thus may occur in proteins. However, in the absence of further evidence, the simplified assumption will be made in the following discussion that the number of open peptide chains is equal to the total number of chains in the molecule. Even if this is not true, it is likely that the number of open chains has a similar significance in relation to structure and immunological specificity.

The marked structural difference between the human adult and foetal haemoglobins was to be expected in view of the other differences that have been observed, e.g. rate of alkali denaturation (Brinkman & Tonxis, 1935), immunological specificity (Darrow, Nowakovsky & Austin, 1940), electrophoretic mobility and sedimentation rate (Andersch *et al.* 1944). It would seem clear that the difference in the two molecules arises from dissimilarity of chemical structure, and not from a physical association or dissociation of the same chemical units.

It is also apparent from lysine content, terminal amino-acid residue, molecular weight and other properties that myoglobin is chemically quite distinct from haemoglobin of the same species.

Of the normal haemoglobins, those from the cow, sheep and goat have four chains/molecule, two with a terminal valyl residue and two with a terminal methionyl residue. Roche, Michel & Schiller (1944) claim to have isolated the phenyl hydantoin of phenylalanine from a hydrolysate of sheep globin which had been treated with phenyl *iso*cyanate, but we could find no evidence of a terminal phenylalanyl residue. The human haemoglobin has five chains, each with a terminal valyl residue, and the horse and donkey haemoglobins six chains with terminal valyl residues. Boyes-Watson & Perutz (1943), from an X-ray study of a single crystal of horse haemoglobin, deduced that the molecule probably consisted 'of four equal and parallel layers of scattering matter'. This need not conflict with our results as the lengths of the six chains are not known, and it is possible that they could be grouped or folded in such a manner as to give four such equal layers.

The results given in Table 6 were obtained on preparations of DNP-globin from which the haem had been removed either before or after the reaction with FDNB. No difference was found in the numbers of the terminal amino-acids, thus proving that the haem is not linked to the globin through an α-amino group. The accuracy of the method is such that a difference of one or two in the number of free lysine ε-amino groups could not be detected with certainty. The possibility of the linkage of the haem through this group must therefore remain.

The lysine contents of three of the haemoglobins used have been determined by Dr Macpherson in this laboratory and are given in Table 8. Comparison of the number of free ϵ-amino groups as measured by this method and the lysine contents shows good agreement. It is therefore probable that all these groups are free in these proteins, and that no branching or cross linking of the peptide chains occurs through them.

Table 8. *Lysine contents of haemoglobins*

Haemoglobin	Mol. wt.	Number of lysine residues/mol.	Number of free lysine ϵ-amino groups/mol.
Horse	66,000	39	40
Sheep	66,000	45	47
Horse myoglobin	17,000	19	20

Relation of molecular structure to immunological specificity. An extensive study of the immunological behaviour of the haemoglobins has been made by Hetkoen & Schulhof (1923), Hetkoen & Boor (1931) and Heidelberger & Landsteiner (1923). The results show that in their serological properties the haemoglobins are similar to the other pure proteins that have been examined, and the conclusions may be summarized as follows: (1) Proteins having the same function in genealogically related animals cross-react with each other. (2) The serological differences between such proteins increase with the distance apart in the zoological scale, but some similarity persists even in proteins from distantly related animals. (3) The specificity of a protein is altered by certain types of denaturation, but some species specificity remains.

Our results are in parallel with the first conclusion; the haemoglobins from the horse and donkey, which cross react strongly, both have the same terminal residues and number of open chains. Those from the cow, sheep and goat fall into another group, both by end-group assay and immunological behaviour, whereas the human adult and foetal haemoglobins are distinct from each other and from the first two groups in both respects. With regard to the second conclusion it is interesting to note that all the haemoglobins examined have at least one chain with a terminal valyl residue, but myoglobin, shown by Hetkoen (1928) to be immunologically distinct from haemoglobin in the dog, has one chain with a terminal glycyl residue. It should be noted that its molecular weight and amino-acid composition are also markedly different.

Landsteiner's study of azo proteins originated the idea of small determinant groups which he designated haptenes. According to this concept certain polar groups in the native protein molecule would function as haptenes and the specificity of the molecule would be the result of their net action. The effect of denaturation by heat, dilute acid or dilute alkali (Macpherson & Heidelberger, 1945), contrary to denaturation by urea or guanidine (Erickson & Neurath, 1943), produces a drastic alteration in the specificity of the protein. This presumably implies that the configuration or folding of the peptide chains within the molecule plays an important part in determining the specificity. This apparently conflicting evidence can best be reconciled by assuming that the haptene-functioning parts of the molecule are influenced by the configuration of the molecule. The determinant groups may arise by the folding of the chain to give a specific array of polar side-chain residues which in the unfolded chain are distantly spaced. Such an array would be dependent on the configuration of the molecule. The disordering of the polar groups, as might occur in certain types of denaturation, would disrupt the specificity-determining parts of the molecule and would lead to a marked change in immunological behaviour. In such a completely denatured protein, where the configuration is believed to be random, the specificity would then depend only on the relative numbers of the different amino-acids and their order along the peptide chain. The haemoglobins of allied species have been shown to have a similar number of open peptide chains and probably have a similar amino-acid composition. It would thus be expected that denaturation would increase the cross reactions of the haemoglobins from related species whilst the distinction between haemoglobins with different numbers of chains/molecule would be maintained. Evidence on this point could not be found in the literature and it is hoped to investigate this further.

Our results suggest a relationship between the terminal residues and immunological specificity. That the free amino groups are part of the determinant groups seems unlikely from the work with toxins and toxoids (Pappenheimer, 1938), and it seems probable that the number of chains in the molecule plays a more predominant role in the immunological behaviour, and this suggests the importance of gross structure in addition to amino-acid composition in determining the specificity of a protein. Our results bring out chemical similarities as well as differences between the haemoglobins, a point not previously shown. The immunological differences that exist between closely allied species can no doubt be accounted for by slight variations in configuration. The results are therefore in agreement with the suggestion as to the basis of the specificity of native proteins outlined above, but much more evidence will be required before more definite conclusions can be drawn.

SUMMARY

1. Several DNP-amino-acids have been prepared and characterized.

2. Several modifications of the method of measuring the free amino groups of proteins with 1:2:4-fluorodinitrobenzene are described.

3. The method has been applied to several haemoglobins. Horse and donkey haemoglobins contain six terminal valyl residues/molecule, adult human haemoglobin five terminal valyl residues, and cow, sheep and goat haemoglobins two terminal valyl and two terminal methionyl residues. If a molecular weight of 66,000 is assumed, human foetal haemoglobin contains 2·6 terminal valyl residues. Horse myoglobin has one terminal glycyl residue/molecule of molecular weight 17,000. The ϵ-amino groups of the lysine residues are free in the above proteins.

4. The significance of these results in relation to the structure of the haemoglobins and the correlation between structure and immunological specificity of proteins is discussed.

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Metabolism of Acetoacetate in Animal Tissues. 2

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The first paper on this subject (Krebs & Eggleston, 1945) was concerned with the anaerobic reactions of acetoacetate. It was shown that in isolated sheep heart two rapid reactions of acetoacetate occur:

acetoacetate + L-malate
= L-β-hydroxybutyrate + oxaloacetate,
acetoacetate + α-ketoglutarate
= L-β-hydroxybutyrate + succinate + CO_2.

These two reactions account for the *anaerobic* metabolism of acetoacetate in sheep heart muscle: all the acetoacetate removed was recovered, within the limits of error, as β-hydroxybutyrate. The present paper deals with the *aerobic* reactions of acetoacetate. Whilst this work was in progress some of its problems were solved by Buchanan, Sakami, Gurin & Wilson (1945) with the help of isotopic carbon. These workers showed beyond doubt that the carbon

skeleton of acetoacetate enters the tricarboxylic cycle.

The main contributions of the present paper are measurements of the rate of the oxidation of acetoacetate in various tissues and the effect on these rates of the physiologically occurring dicarboxylic acids, as well as quantitative estimations of the products of acetoacetate metabolism. The results are in agreement with the conception that acetoacetate is oxidized through the tricarboxylic cycle.

METHODS

Tissue preparations

Suspensions of minced tissue were used in the same way as in the previous experiments (Krebs & Eggleston, 1945), unless otherwise stated. The metabolic activities were interrupted after a specified period of incubation by addition of 0·25 vol. of 2N-HCl.

Analytical procedures

Changes in the previous methods concerned the following points.

Determination of pyruvate in the presence of oxaloacetate. Oxaloacetate was not found in significant quantities when heart or kidney were incubated with this substrate under anaerobic conditions (unless the period of incubation was very short). Aerobically, however, oxaloacetate was often present, presumably because it was continuously reformed from malate. The problem therefore arose of determining pyruvate in the presence of oxaloacetate. Carboxylase decarboxylates oxaloacetate under the conditions used for the determination of pyruvate, but the amounts of CO_2 split off are variable. Under the standard conditions for the determination of pyruvate the initial rate of CO_2 formation from oxaloacetate was about 15% of that from pyruvate. The rate of CO_2 production gradually slowed down and the reaction practically stopped after 6 hr. when about 1·2 moles CO_2 had been formed/mole oxaloacetate (Table 1). The slowing down of the rate was not due to inactivation of carboxylase, for addition of pyruvate showed that the enzyme was still highly active. In more concentrated yeast extracts (4 ml. yeast extract, 0·2 ml. 3M-acetate buffer, pH 5·0; 0·1 ml. 0·1M-substrate solution) the total yield of CO_2 from oxaloacetate was only 0·6 mole/mole. These observations indicate that there are other reactions of oxaloacetate besides decarboxylation, the extent of which varies with the conditions. Neuberg & Karczag (1911) found that their yeast suspension liberated 2 moles of CO_2 from 1 mole oxaloacetate.

Table 1. *Decarboxylation of oxaloacetate by yeast extract*

(Yeast extract from English brewer's yeast (see Krebs & Johnson, 1937). Conditions for the manometric determination of CO_2 evolution according to Krebs & Eggleston (1945). The main compartment of each cup contained 3 ml. water; 0·1 ml. 0·1M-substrate; 0·2 ml. 3M-acetate buffer, pH 5·0; side arm, 1 ml. carboxylase. The data given are corrected for a small CO_2 evolution from yeast without substrate.)

Time after carboxylase addition (min.)	Substrate: Pyruvate (224 μl.) CO_2 evolved (μl.)	Substrate: Oxaloacetate (224 μl.) CO_2 evolved (μl.)
2	84	11·5
4	160·5	25·5
8	217·5	61·5
12	224	89
20	224	136
40	224	211
120	227	251
180	—	259
240	—	269

The following procedure was proved satisfactory in overcoming the difficulties created by the irregular yields of CO_2 from oxaloacetate: A measured fraction of the acidified tissue suspension containing 0·4N-HCl was heated for 15 min. in a water bath at 100° and the pH was then adjusted to 5·0 by addition of 3M-sodium acetate. This treatment converts oxaloacetate quantitatively into pyruvate and CO_2 whilst not destroying preformed pyruvate. The determination of pyruvate in the solution thus yielded the sum of pyruvate and oxaloacetate. In another fraction of the tissue suspension oxaloacetate was determined by the aluminium sulphate method (Krebs & Eggleston, 1945). The difference represented the preformed pyruvate.

Determination of oxaloacetate in the presence of pyruvate and acetoacetate. The manometric method previously used was based on the observation that multivalent cations specifically decarboxylate β-keto-dicarboxylic acids (Krebs, 1942). In the course of the present work it was found that acetoacetate reacts also if pyruvate or other α-ketonic acids are present (Table 2). In other words, α-ketonic acids have a catalytic effect on the decomposition of acetoacetate by aluminium ions. It is very probable that this is due to the formation, by aldol condensation, of a dicarboxylic β-ketonic acid from the α-ketonic acid and acetoacetate, e.g.

$$\mathrm{COOH.CO.CH_3 + CH_3.CO.CH_2.COOH} \rightleftharpoons \mathrm{COOH.C(OH)(CH_3).CH_2.CO.CH_2.COOH}.$$

In general the effect of α-ketonic acids was not great because of the low concentration of these substances in the solutions to be tested. The magnitude of the effect was estimated by extrapolation. Warburg cups were filled as previously described for the determination of oxaloacetate, and readings were taken for a period of 60 min. after the addition of $Al_2(SO_4)_3$. Under the conditions of the test oxaloacetate was quantitatively decomposed after 40 min. The CO_2 evolution which occurred between 40 and 60 min. was taken as due to the decomposition of acetoacetate and twice this value was deducted from the 40 min. reading to obtain the amount of oxaloacetate in the solution. This correction factor was probably slightly too high as the rate of CO_2 evolution from mixtures of acetoacetate and α-ketonic acid slightly increased with time (Table 2).

Acetoacetate does not seem to form appreciable quantities of such a condensation product with a second molecule of acetoacetate as long as the concentration of acetoacetate is low (0·005M). At higher concentrations (0·25M final concentration) Al ions accelerate the decarboxylation: without Al^{+++}, 0·25% was decomposed in 60 min., and with Al^{+++} 2·1% (conditions as stated in Table 2).

Table 2. *Evolution of CO_2 from mixtures of acetoacetate with α-ketonic acids in the presence of aluminium salts*

(Conditions as given by Krebs & Eggleston (1945) for the determination of oxaloacetate. Each cup contained 0·2 ml. 0·1 M-acetoacetate in addition to the substrate stated below. Concentration of added substrate solutions 0·1 M.)

	Substrates added						
Time after Al^{+++} addition (min.)	0	0·05 ml. pyruvate	0·2 ml. pyruvate	1·0 ml. pyruvate	0·05 ml. α-keto-glutarate	0·1 ml. α-keto-glutarate	0·2 ml. α-keto-glutarate
	CO_2 evolved (μl.)						
10	0	0	19	80	0	7	32
20	0	5	44	147	0	14	64
40	0	17	93	232	0	29	123
60	0	26	126	272	3·5	46·5	165
120	4	62	188	320	17	88·5	249
240	14	86	239	343	—	—	—

Table 3. *Substances simulating β-hydroxybutyrate in the method of Van Slyke* (1917) *and of Edson* (1935)

Substance	Amount added (mg.)	'β-Hydroxybutyrate' formed (mg.)	Yield (mg. β-hydroxy-butyrate/100 mg. substance tested)
L-Lactic acid	18	6·31	35·1
	9	2·80	31·1
	4·5	0·99	22·0
	2·25	0·04	1·78
	1·125	0·04	3·56
L-Malic acid	26·8	2·03	7·6
	13·4	1·14	8·5
	6·7	0·21	3·1
Fumaric acid	23·2	4·65	20·0
	11·6	1·80	15·5
	5·8	0·48	8·3
α-Ketoglutaric acid	29·2	0·287	0·98
	14·6	0·093	0·61
	7·3	0·032	0·23
Pyruvic acid	17·6	0·505	2·87
Citric acid	38·4	0·884	2·3
Succinic acid	23·6	Nil	Nil

Determination of β-hydroxybutyrate. The method previously used (Edson, 1935; Krebs & Eggleston, 1945) was modified in the following points in order to increase accuracy. Instead of 1 ml. 5% potassium dichromate, 2·5 ml. were added. The precipitate formed after the dichromate oxidation was filtered off when the solution had cooled to room temperature. Sintered glass funnels (3G3) allowing about 5–7 ml. fluid to filter/min. were used. Under the conditions of the determination 1·0 ml. 0·01 N-iodine were equivalent to 0·166 mg. β-hydroxybutyric acid in the filtrate.

Several substances present in the solution were found to simulate small quantities of β-hydroxybutyrate. Van Slyke (1917, 1929) already found this for lactate provided it was present in fairly large quantities. Data on the errors caused by various substances are given in Table 3. The concentrations of interfering substances were known and the data in Table 3 could thus be used for correcting results. The correcting factor was generally below 5% of the amounts of β-hydroxybutyrate as the quantities of the interfering substances in the sample were small, viz. lactic acid between 0·3 and 1·2 mg.; malic acid between 0·5 and 2 mg.; fumaric acid between 0·5 and 1 mg.

The errors were of the same order in pure solutions and in mixtures with β-hydroxybutyrate. Attempts to increase the specificity of the method by separating β-hydroxybutyrate from the interfering substances through chromatography were unsuccessful.

Fumaric acid was in a number of cases determined by the manganometric method of Straub (1935).

Citric acid. Water was used instead of dioxan to dilute the final alkaline Na_2S solution to the required volume in the colorimetric determination. The colour was found to be more stable in this medium than in aqueous solutions of commercial 'pure' dioxan.

RESULTS

Comparative rates of reduction and oxidation of acetoacetate

In Table 4, experiments on sheep heart and sheep kidney cortex are recorded in which the amounts of acetoacetate removed and β-hydroxybutyrate

Table 4. *Amounts of acetoacetate removed and β-hydroxybutyrate formed*

(All data refer to 4 ml. suspension; acetoacetate 'oxidized' refers to the amounts removed which are not accounted for by the formation of β-hydroxybutyrate.)

Exp. no.	Tissue and medium	Acetoacetate added (μl.)	Other substrates added (μl.)	Period of incubation (min.)	Changes in N_2: Acetoacetate removed (μl.)	Changes in N_2: β-Hydroxybutyrate formed (μl.)	Changes in N_2: Acetoacetate 'oxidized' (μl.)	Changes in O_2: Acetoacetate removed (μl.)	Changes in O_2: β-Hydroxybutyrate formed (μl.)	Changes in O_2: Acetoacetate 'oxidized' (μl.)
1	Sheep heart, minced	1692	—	60	—	—	—	910	527	383
	1 part +9 parts phosphate	1692	Oxaloacetate (224)	60	—	—	—	1110	646	464
	saline	1692	Oxaloacetate (112)	60	—	—	—	1032	580	452
2	Ditto	1760	—	60	377	331	46	1062	782	280
		1760	Fumarate (448)	60	1215	1218	~Nil	1245	1040	205
3	Ditto	846	—	40	—	—	—	435	406	29
		846	Fumarate (896)	40	—	—	—	656	620	36
		846	Oxaloacetate (896)	40	—	—	—	604	561	43
4	Ditto	850	—	20	—	—	—	310	304	6
		850	—	40	—	—	—	493	349	144
		850	—	120	—	—	—	492	184	308
		850	Oxaloacetate (896)	20	—	—	—	569	375	194
		850	Oxaloacetate (896)	40	—	—	—	764	409	355
		850	Oxaloacetate (896)	120	—	—	—	691	156	535
5	Ditto	878	—	40	—	—	—	558	325	233
		878	Oxaloacetate (896)	40	—	—	—	691	417	274
		878	Fumarate (896)	40	—	—	—	706	427	279
6	Ditto	1740	—	30	—	—	—	410	304	106
		1740	Oxaloacetate (896)	30	—	—	—	825	521	304
		1740	Fumarate (896)	30	—	—	—	759	573	186
7	Sheep kidney cortex minced	1856	—	40	386	366	20	653	227	426
	1 part +9 parts phosphate	1856	Fumarate (896)	40	751	778	~Nil	656	309	347
	saline	1856	Oxaloacetate (896)	40	651	485	166	656	279	377
8	Ditto	1760	—	60	360	322	38	355	175	180
		1760	Fumarate (448)	60	680	658	22	550	320	230
9	Ditto	872	—	60	—	—	—	394	182	212
		872	Fumarate (896)	60	—	—	—	642	466	176
		872	Oxaloacetate (896)	60	—	—	—	604	366	238
10	Ditto	829	—	60	—	—	—	204	127	77
		829	Fumarate (896)	60	—	—	—	359	289	70
		829	Oxaloacetate (896)	60	—	—	—	341	158	183
11	Ditto	1285	—	90	—	—	—	647	229	418
		1285	Oxaloacetate (1344)	90	—	—	—	1084	509	575
12	Ditto	1647	—	30	—	—	—	220	161	59
		1647	Oxaloacetate (1792)	30	—	—	—	622	287	335
13	Ditto	847	Oxaloacetate (896)	20	—	—	—	261	181	80
		847	Oxaloacetate (896)	40	—	—	—	436	274	162
		847	Oxaloacetate (896)	80	—	—	—	613	239	374
		847	Oxaloacetate (896)	160	—	—	—	630	212	418
14	Ditto	1871	—	40	374	236	138	—	—	—
		1871	Oxaloacetate (1792)	40	661	436	225	—	—	—
15	Ditto	942	Oxaloacetate (448)	30	244	183	61	324	164	160
16	Sheep kidney cortex minced 1 part+2·6 parts NaCl-$NaHCO_3$ (medium of Breusch, 1943 *b*)	888	Oxaloacetate (896)	20	588	421	167	583	433	150
17	Ditto	848	Oxaloacetate (1792)	30	673	501	172	—	—	—
		848	Oxaloacetate (1792)+pyruvate (820)	30	704	488	216	—	—	—
18	Ditto	1742	Oxaloacetate (3584)	30	1406	1081	325	—	—	—
		1742	Oxaloacetate (3584)+pyruvate (1650)	30	1406	1046	360	—	—	—
19	Sheep kidney cortex minced 1 part +7 parts phosphate-$NaHCO_3$ saline	890	Oxaloacetate (1792)	15	357	283	74	—	—	—
		890	Oxaloacetate (1792)	30	490	438	52	—	—	—
		890	Oxaloacetate (1792)	60	603	620	Nil	—	—	—

formed are compared. The data indicate the following:

(1) Anaerobically, as in the previous experiments (Krebs & Eggleston, 1945), almost all acetoacetate removed in sheep heart was accounted for by the formation of β-hydroxybutyrate. A small excess was sometimes found when no additional substrate was added, but this was probably due to the non-enzymic ketone decomposition. No correction was applied for ketone decomposition, and as the average acetoacetate concentration during the experiment was usually higher when no further substrate was added (because the reduction was slower), the error in the calculation of the amounts 'oxidized' arising from ketone decomposition was larger in the absence of additional substrates. In sheep kidney the quantities of acetoacetate 'oxidized' anaerobically exceeded the experimental error if oxaloacetate was present (Exp. nos. 7, 14, 16, 17, 18).

(2) Aerobically a large proportion of the acetoacetate removed was still due to reduction to β-hydroxybutyrate, especially in short experiments in accordance with results published by Quastel & Wheatley (1935). On prolonged incubation, however, the amount of 'oxidized' acetoacetate gradually increased, whilst the amounts of β-hydroxybutyrate, after a rapid increase during the early periods of incubation, showed a fall in the later periods. It thus appears that in the early stages, when the acetoacetate concentration is high, the system acetoacetate $\rightleftharpoons$ β-hydroxybutyrate reacts predominantly from left to right. Later, when most of the added acetoacetate has disappeared the reverse reaction predominates (Exps. 4 and 13).

(3) The amounts of acetoacetate oxidized aerobically were increased in many experiments by oxaloacetate or fumarate, both in heart muscle and in kidney. This may be taken as supporting the view that the four-carbon dicarboxylic acids play some part in the oxidation of acetoacetate (Quastel & Wheatley, 1935; Edson & Leloir, 1936; Breusch, 1943*a*, *b*; Wieland & Rosenthal, 1943; Lehninger, 1946), a conclusion which is not invalidated by the fact that the effects did not occur in every experiment (since the tissues are known to contain considerable quantities of precursors of oxaloacetate).

(4) In heart muscle, the initial, that is maximum, rate of aerobic reduction of acetoacetate calculated in terms of $Q_{\beta\text{-hydroxybutyrate}}$, assuming the dry weight to be 1/5 of the wet weight, was 11·4 without oxaloacetate, 14·1 with oxaloacetate (Table 4, Exp. 3). The maximum rates of oxidation without oxaloacetate (same exp., period 20–40 min.) in heart were 5·2 and 7·3 with oxaloacetate. In this tissue the maximum rates of reduction were thus about twice those of oxidation. In kidney cortex the corresponding rates of reduction were 4·0 without and 7·2 with oxaloacetate, the rates of oxidation 1·5 without and 8·4 with oxaloacetate (Table 4, Exp. 12). In kidney the maximum rates of oxidation and reduction in the presence of oxaloacetate therefore were of the same order.

Products of acetoacetate removal

Examples giving comprehensive analyses of the products of acetoacetate metabolism are shown in Table 5. The comparison of the yields of the various substances on the addition of oxaloacetate, with those found on addition of both oxaloacetate and acetoacetate, shows that acetoacetate caused an increase in the yields of citrate and of succinate,

Table 5. *Acetoacetate and oxaloacetate in sheep kidney*

(48 ml. suspension containing 1 part minced kidney cortex and 9 parts phosphate saline pH 7·4. All quantities are in μl. and refer to 48 ml. suspension. The three experiments were carried out on different kidneys.)

	Exp. 1 N_2 (40 min. incubation)			Exp. 2 O_2 (30 min. incubation)			Exp. 3 O_2 (30 min. incubation)	
Substrates added:								
Acetoacetate	22,460	—	22,460	19,760	—	19,760	—	12,140
Oxaloacetate	—	21,500	21,500	—	21,500	21,500	21,500	21,500
Substances found after incubation:								
Acetoacetate	17,970	~0	14,520	17,360	—	12,300	~0	6,230
β-Hydroxybutyrate	2,830	~0	5,210	1,670	—	3,410	~0	2,915
Oxaloacetate	~0	1,210	1,850	—	2,670	4,400	1,640	2,770
Pyruvate	~0	4,070	4,750	—	—	—	550	1,400
Succinate	~0	1,270	2,005	—	320	1,360	240	342
α-Keto-(plus α-hydroxy)-glutarate	1,110	1,605	1,655	1,275	2,160	550	725	~0
Fumarate	—	2,765	880	—	—	—	—	—
L-Malate	~0	4,605	3,155	—	3,640	3,240	2,590	2,500
Citrate	10	156	1,690	—	1,530	3,240	1,740	3,820
Calculated changes:								
Acetoacetate reduced	2,830	—	5,210	1,670	—	3,410	—	2,915
Acetoacetate 'oxidized'	1,660	—	2,730	730	—	4,050	—	2,995

whilst the yields of α-keto(plus α-hydroxy)glutarate were decreased. On balance, less oxaloacetate disappeared when acetoacetate was present. The effects of oxygen on the yields were altogether not very great, the most notable difference being the higher yield of citrate in O_2.

The observations on the differences in O_2 and N_2 are in general in accordance with the view that aerobically the same reactions occur as anaerobically with certain additional reactions, namely, some of the oxidative steps of the tricarboxylic cycle and the oxidation of β-hydroxybutyrate to acetoacetate. The experiments do not allow any conclusions on the mechanism of the formation of citrate in the presence of oxaloacetate and acetoacetate.

A number of similar experiments were carried out (before the tracer work of Buchanan *et al.* (1945) decided the question conclusively) with the intention of throwing light on the mechanism of the increased citric acid formation in the presence of oxaloacetate and acetoacetate, especially on the conflicting views expressed by Breusch (1943*a*, *b*) and by Wieland & Rosenthal (1943) on the one hand and by ourselves (Krebs & Eggleston, 1945) on the other. If our earlier view that acetoacetate does not take a direct part in the formation of citrate were to apply also to aerobic conditions, the sum of the reaction products arising from oxaloacetate, i.e. the acids of the tricarboxylic cycle, including pyruvic acid, would be limited by the amount of carbon added in the form of oxaloacetate. Each molecule of tricarboxylic acid, α-keto- and α-hydroxy-glutarate and the bulk of the succinate molecules would require 2 molecules of oxaloacetate and each molecule of fumarate, malic and pyruvate would require 1 molecule of oxaloacetate. According to Breusch and Wieland & Rosenthal, only 1 molecule of oxaloacetate would be needed for each molecule of acid because acetoacetate would provide part of the carbon skeleton of the tricarboxylic acids, of α-keto- and α-hydroxyglutarate and of part of succinate.

Calculations based on five experiments are shown in Table 6. According to the last column more 'oxaloacetate equivalents' were recovered in some experiments than were added; this discrepancy was always greater when acetoacetate was present. The latter observation argues against our previous contention and suggests that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle. The results in Table 6 however can hardly be taken as decisive, as the effects in question are relatively small and the errors of analysis and uncertainties in the calculations may be considerable; the errors are the aggregate from six analyses and the uncertainties arise from the question how much succinate is formed by reduction and how much by oxidation from oxaloacetate. In aerobic experiments, moreover, some succinate was presumably oxidized to fumarate though this reaction was inhibited during the initial stages of incubation owing to the inhibition of succinic dehydrogenase by oxaloacetate.

This line of approach was not continued when the results of the tracer work of Buchanan *et al.* (1945)

Table 6. *Comparison of the amounts of oxaloacetate added and the amounts of substances arising from oxaloacetate*

(The quantities of metabolites arising from oxaloacetate are expressed in 'oxaloacetate equivalents'. For the purpose of calculating the equivalents, the tricarboxylic acids, α-keto- and α-hydroxy-glutaric acids are taken as requiring two oxaloacetate equivalents/molecule, fumaric, malic and pyruvic acids as requiring one equivalent. Succinic acid was taken as requiring two equivalents because in the presence of acetoacetate most of the succinate is known to be formed by oxidation from α-ketoglutarate (Krebs & Eggleston, 1945). All data refer to 48 ml. minced tissue suspension containing 1 part of tissue for 9 parts of phosphate saline.)

Tissue	Substrates added (μl.)	Aerobic or anaerobic	Period of incubation (min.)	Oxaloacetate removed (μl.)	Acetoacetate 'oxidized' (μl.)	Oxaloacetate equivalents recovered (μl.)	Balance of oxaloacetate (μl.)
Sheep kidney cortex	(*a*) Oxaloacetate (21,500)	N_2	40	20,290	—	17,530	+2,760
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,460)			19,650	2,730	19,850	− 200
Sheep heart	(*a*) Oxaloacetate (21,500)	N_2	40	21,170	—	22,540	−1,370
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,750)			19,320	2,330	23,530	−4,210
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	N_2	30	10,280	—	11,480	−1,200
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,320	1,460	10,820	−1,500
Sheep heart	(*a*) Oxaloacetate (21,500)	O_2	40	17,450	—	15,210	+2,240
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,270)			16,370	4,632	13,700	+2,670
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	O_2	30	10,480	—	9,980	+ 500
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,850	3,850	13,090	−3,240

became available, as this work provided direct evidence for the participation of acetoacetate in the formation of the tricarboxylic acids and α-ketoglutarate. The data reported in their paper are in accordance with this conception.

Metabolism of DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

If it is agreed that acetoacetate provides part of the tricarboxylic acid and α-ketoglutarate molecule, there remains the problem to be solved of the intermediary stages of the formation of these substances from oxaloacetate and acetoacetate.

The rapid reduction of acetoacetate to β-hydroxybutyrate, even in the presence of O_2, suggests the possibility that the reduction is a stage in the final breakdown of acetoacetate. This step might then be followed by an oxidation of β-hydroxybutyrate to 1:2-dihydroxybutyric acid (see Weil-Malherbe, 1938; Haarmann & Schroeder, 1938; Hoff-Jørgensen, 1940). There are apparently conflicting statements in the literature on the metabolism of 1:2-dihydroxybutyric acids. Weil-Malherbe (1938) found that kidney does not remove the erythro form, but oxidizes the threo form to α-hydroxyacetoacetate, a reaction to which he attaches no physiological significance because α-hydroxyacetoacetate is not further metabolized. Hoff-Jørgensen reported on the other hand that the erythro form is rapidly destroyed after intravenous injection, whilst the threo form was stable under the same conditions. In view of these discrepancies the question was re-examined. The results essentially confirm Weil-Malherbe's findings (Table 7). The erythro form was not attacked by sheep kidney; the threo form caused a slight increase in the O_2 uptake and a slight decrease of the concentration equivalent to 0·8 ml. 0·01 N-iodine. This is accounted for, according to Weil-Malherbe, by oxidation to α-hydroxyacetoacetate. Thus neither the threo nor the erythro forms can be intermediates in the removal of ketone bodies by sheep kidney cortex.

Sequence of formation of α-ketoglutaric and tricarboxylic acids

From Ochoa's work (1945) it is known that the reaction *iso*citrate → α-ketoglutarate + CO_2 is reversible in some tissues. Hence the system

$$\text{citrate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \textit{cis}\text{aconitate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \textit{iso}\text{citrate} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + CO_2 \quad (1)$$

is completely reversible and the simultaneous occurrence of α-ketoglutarate and the tricarboxylic acids, as the result of an interaction between oxaloacetate and acetoacetate (or between other metabolites), may be due to the system (1) reacting from left to right, or in the reverse order (see also Wood, 1946). The following experiments were designed to throw light on this question. If the sequence is

$$\text{oxaloacetate} + \text{acetoacetate} \rightarrow \text{tricarboxylic acid} \rightarrow \alpha\text{-ketoglutarate} \quad (2)$$

tricarboxylic acids should yield α-ketoglutarate at least as rapidly as a mixture of oxaloacetate and acetoacetate. Experiments in Table 8 show that this is the case in sheep kidney. Various substrates and substrate combinations were added to malonate-poisoned tissue, and the sum of α-ketoglutarate and succinate was measured after aerobic incubation. The reductive formation of succinate from fumarate is small under the conditions of the experiment, and the sum of α-ketoglutarate and succinate may therefore be taken as a measure of the formation of α-ketoglutarate.

Table 7. *Metabolism of* DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline. Incubation 90 min. Determination of dihydroxybutyric acid with periodate according to Weil-Malherbe (1938); the iodine equivalent of the added substrate was 16 ml. 0·01 N-iodine at the beginning; 1 ml. 0·01 N-I_2 = 112 μl. DL-dihydroxybutyrate.)

Substrate added (final conc. 0·02 M)	Q_{O_2}	Iodine equivalent (ml. 0·01 N-I_2) of 4 ml. suspension: At end of incubation	Corrected for 'blank'	Change during incubation
—	−12·4	5·9	—	—
Erythro form	−12·3	23·9	18·0	+2·0
Threo form	−13·0	21·1	15·2	−0·8

It will further be seen from Table 8 that pyruvate, acetate and acetoacetate cause some formation of α-ketoglutarate. In Exp. III the increase caused by combination of pyruvate + acetate and pyruvate + acetoacetate is somewhat larger than the sum of the individual increases, but in every case the tricarboxylic acids yield α-ketoglutarate more rapidly than other substrates. The results are thus in accord-

ance with the sequence (2). On the other hand, the following experiments exclude the sequence oxaloacetate + acetoacetate → α-ketoglutarate → tricarboxylic acid. Suspension of sheep kidney was anaerobically incubated with various substrate combinations in a medium containing 0·02 M-$NaHCO_3$ and in equilibrium with 5 % CO_2 in the gas phase. Table 9 shows that α-ketoglutarate yields only traces of citrate, less than 10 % of the amount formed from a combination of oxaloacetate and acetoacetate.

measured with the help of a procedure developed in this laboratory (Krebs & Eggleston, 1944). The interpretation of these measurements is based on a characteristic property of aconitase: if citrate arises in the presence of aconitase (from a precursor other than *cis*aconitate or *iso*citrate) 10·5 % will gradually be converted into a mixture of *cis*aconitate and *iso*citrate (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943). If, however, *cis*aconitate arises according to scheme (3) a large proportion (up to 25 %)

Table 8. *Rate of formation of α-ketoglutarate from various precursors in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline pH 7·4 containing 0·005 M-malonate. O_2. Final substrate concentrations in Exp. 1: 0·01 M except oxaloacetate which was 0·02 M; in Exps. II–IV 0·02 M. The data refer to 4 ml. suspension; 'α-ketoglutarate + succinate' is the amount of succinate found in the solution after treatment with acid permanganate.)

		α-Ketoglutarate + succinate formed (μl.) in presence of										
Exp.	Period of incubation (min.)	Nil	Citrate	*Cis*-aconitate	Aceto-acetate	Citrate + aceto-acetate	Pyru-vate	Pyruvate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Oxalo-acetate + pyruvate	Acetate	Pyruvate + acetate
	30	59	463	—	87	471	91	110	342	464	356	—	—
II	40	91	492	405	—	—	183	—	—	—	—	93	235
III	40	46	398	—	88	—	109	188	—	—	—	80	182
IV	20	90	—	—	103	—	137	147	—	—	—	—	—
	40	77	—	—	109	—	200	236	—	—	—	—	—

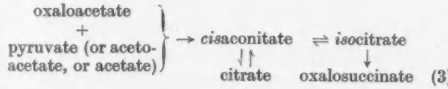
Table 9. *Rate of citrate formation in sheep kidney cortex from various precursors*

(Minced tissue suspended in 9 parts phosphate bicarbonate saline. 5 % CO_2 in N_2. Substrate concentrations: 0·02 M except acetoacetate which was 0·01 M. Period of incubation 30 min. The data refer to 4 ml. suspension.)

Substrates added	Nil	α-Keto-glutarate	α-Keto-glutarate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Pyruvate + aceto-acetate	α-Keto-glutarate + oxalo-acetate + aceto-acetate
μl. citrate formed	Nil	23	23	119	298	23	258

Order of formation of tricarboxylic acids

It is now generally believed that the sequence of formation of the tricarboxylic acids in the tricarboxylic cycle is as follows:



(3)

This scheme rests chiefly on the work of Wood *et al.* (1941), Evans & Slotin (1941), Buchanan *et al.* (1945) and Weinhouse *et al.* (1945) which showed that citrate cannot be an intermediate on the main path leading from oxaloacetic acid to oxalosuccinic acid. Further evidence supporting this scheme is supplied by the experiments described in this section in which the formation of citrate on the one hand and of the sum of *cis*aconitate and *iso*citrate on the other was will be converted into *iso*citrate during the early stages of the reaction. In the later stages *iso*citrate (if it is not dehydrogenated) may be reconverted into *cis*aconitate and thence into citrate until an equilibrium containing 89·5 % citrate is established (Martius, 1938; Krebs & Eggleston, 1943). Therefore, (*a*) if citrate were the primary product of interaction between oxaloacetate and its partner, the sum of *cis*aconitate and *iso*citrate could never exceed 10·5 % of the tricarboxylic acids present (but may be less if the reaction is interrupted before equilibrium is established, or if *iso*citrate is dehydrogenated more rapidly than it is formed); (*b*) if *cis*aconitate is produced according to scheme (3) the sum of *cis*aconitate and *iso*citrate may exceed 10·5 % of the total tricarboxylic acids present provided that the reaction is interrupted before equilibrium is established (but may be less if *iso*citrate is dehydrogenated more rapidly than it is formed).

It follows that if the sum of *cis*aconitate and *iso*citrate is found to exceed the limit of 10·5 % this

may be taken as conclusive evidence against the scheme (4) and as direct support of scheme (3).

$$\left.\begin{matrix}\text{oxaloacetate}\\+\\\text{pyruvate, or}\\\text{acetoacetate or}\\\text{acetate}\end{matrix}\right\} \rightarrow \text{citrate} \rightleftharpoons \textit{cis}\text{aconitate} \rightleftharpoons \textit{iso}\text{citrate} \rightarrow \text{oxalosuccinate} \quad (4)$$

Table 10 shows the results of four experiments in which the tricarboxylic acids were estimated under varying conditions. The incubation periods were relatively short because it was intended to interrupt the reaction before an equilibrium between the three tricarboxylic acids could be established. In several experiments the amounts of *cis*aconitate + *iso*citrate far exceeded the critical figure of 10·5 %, the highest value being 49 %. The fact that the ratio

$$\frac{\textit{cis}\text{aconitate} + \textit{iso}\text{citrate}}{\text{total tricarboxylic acids}}$$

shows great variations with the experimental conditions is not surprising, because this ratio is the resultant of the rates of at least six reactions (indicated by the arrows in scheme 3) each of which varies with the conditions. Variability of the ratio does not invalidate the conclusion that the results of the experiments in Table 10 support scheme 3.

[1] *Note on non-enzymic condensation reactions of oxaloacetate.* Breusch (1941) noted that neutral solutions of oxaloacetate may contain substances which simulate citric acid in analytical methods based on the formation of pentabromoacetone, and that the quantities of interfering substances increased on addition of calcium salts. Our own experiments confirmed this observation, and furthermore showed that substances arise by non-enzymic reactions of oxaloacetate, which behave like *iso*citrate and *cis*aconitate, forming bromo compounds after incubation with aconitase resembling pentabromoacetone. All substances interfering with the determination of citrate and of *cis*aconitate plus *iso*citrate are however quantitatively removed by boiling the acidified solution before treatment with KBr and $KMnO_4$, an obligatory step in the citric acid determination.

Effect of $BaCl_2$ *on the yields of citrate*

Wieland & Rosenthal (1943) and Weinhouse *et al.* (1946) have found that the yields of citrate from a mixture of oxaloacetate and acetoacetate increase if $BaCl_2$ (about 0·01 M) is added to the medium. Data given in Table 11 confirm this finding. Wieland & Rosenthal offer no explanation for the cause of this effect. On the basis of the following scheme one would expect the yield of citrate to rise if a specific inhibitor reduced the rates of (*b*) or (*d*) without major effects on the rates of (*c*). In order to test whether barium ions inhibit specifically (*b*), aconitase (minced sheep heart muscle) was incubated with *cis*aconitate in the presence and absence of

Table 10. *Formation of citrate,* cis*aconitate and* iso*citrate in minced sheep kidney cortex*

(Tissue suspended in 9 parts medium. The data refer to 24 ml. suspension. The media did not contain Ca ions; pH in all cases 7·4.)

Exp. no.	Medium	Gas	Substrate added (final concentrations)	Period of incubation (min.)	Citrate formed (μl.)	*cis*Aconitate + *iso*citrate formed (μl.)	*cis*Aconitate + *iso*citrate as % of total tricarboxylic acids
I	Phosphate saline	O_2	0·01 M-Oxaloacetate	5	128	47	26·8
			0·01 M-Oxaloacetate	10	227	90	28·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	5	229	57	19·9
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	350	268	43·5
II	Phosphate-bicarbonate saline	5% CO_2 in O_2	0·02 M-Oxaloacetate, 0·02 M-pyruvate	30	1600	1560	49·4
			0·02 M-Oxaloacetate, 0·02 M-pyruvate, 0·01 M-acetoacetate	30	3200	~Nil	—
III	Phosphate saline	O_2	0·01 M-Oxaloacetate	10	190	22	10·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	415	47	10·2
IV	Phosphate-bicarbonate saline	5% CO_2 in O_2	0·02 M-Oxaloacetate	20	316	~Nil	—
			0·02 M-Oxaloacetate, 0·02 M-pyruvate	20	580	~Nil	—
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate	20	1000	430	30·1
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate, 0·02 M-pyruvate	20	1240	124	9·1

$BaCl_2$ and the rates of formation of *iso*citrate and citrate were measured.

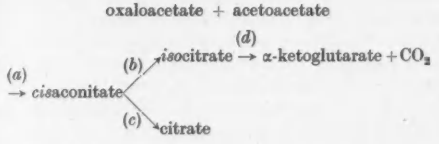


Table 11. *Effects of* $BaCl_2$ *on yields of citrate from oxaloacetate and acetoacetate*

(Minced sheep kidney cortex suspended in 9 parts phosphate-bicarbonate saline. Period of incubation 30 min.; 5% CO_2 in O_2; 0·02 M-oxaloacetate; 0·01 M-acetoacetate. The data refer to 4 ml.)

	Without $BaCl_2$	With 0·02 M-$BaCl_2$
Oxaloacetate removed (μl.)	1310	1200
Acetoacetate reduced (μl.)	72	53
Acetoacetate 'oxidized' (μl.)	258	329
Citrate formed (μl.)	162	344
L-Malate formed (μl.)	189	169

Table 12 demonstrates the phenomenon discovered by Martius (1938) that during the early stages of incubation *iso*citrate is formed more rapidly than citrate, and that *iso*citrate is later reconverted to citrate. $BaCl_2$ (0·02 M) slows down both reactions (*b*) and (*c*) a little. At 10 min. the inhibition of (*b*) is 17%, of (*c*) 28%. Thus the increased yield of citrate from oxaloacetate and acetoacetate cannot be explained by a specific inhibition of reaction (*b*).

With and without barium chloride the maximum yields of *iso*citrate are 23% of the added *cis*aconitate, whilst the final yield is only 5%. Probably owing to the conversion of some *cis*aconitate into the *trans* form during the prolonged incubation the total yield of *cis*aconitate and *iso*citrate was less than that obtained with more powerful aconitase preparations in short-term experiments (Krebs & Eggleston, 1943).

As for effects of barium on reaction (*d*) (the oxidative removal of *iso*citrate), Table 13 indicates that barium ions inhibit the O_2 uptake of muscle without citrate (see Thunberg, 1937), but citrate increases the oxygen uptake also in the presence of Ba and the rate of removal of citrate is only slightly reduced by Ba. This effect is too small to account for the increased yields of citrate and by the method of elimination one is therefore led to the assumption that the rate of *cis*aconitate formation (reaction *a*) is accelerated in the presence of barium ions. Such an

Table 12. *Effect of* $BaCl_2$ *on aconitase*

(4·2 g. frozen minced sheep heart suspended in a total volume of 100 ml.; 0·025 M-phosphate buffer, pH 7·4; 0·1 M-*cis*aconitate = 10 mmol. 40°. At intervals, 10 ml. solutions were removed for analysis and the reaction was stopped by addition of 1·25 ml. glacial acetic acid. *iso*Citrate was determined polarimetrically in 9 ml., citrate in 0·5 ml. The quantities of *iso*citrate and citrate are expressed in mmol.)

	No $BaCl_2$ added		0·02 M-$BaCl_2$	
Time (min.)	*iso*Citrate (mmol.)	Citrate (mmol.)	*iso*Citrate (mmol.)	Citrate (mmol.)
0	0	0	0	0
5	1·17	—	1·03	—
10	1·82	1·70	1·52	1·23
20	2·26	—	1·93	—
30	2·21	3·39	2·17	2·02
40	1·93	—	2·28	—
60	1·42	4·65	2·23	3·12
90	0·91	—	1·94	—
120	0·67	6·61	1·72	4·58
180	0·53	6·58	1·41	5·24
24 hr.	0·56	6·79	0·51	6·80

Table 13. *Effect of* $BaCl_2$ *and the composition of saline medium on* O_2 *uptake and citrate removal in sheep kidney cortex*

(Minced sheep kidney suspended in 9 parts medium. 40°. 60 min. Medium I = phosphate saline, pH 7·4; medium II = 45 ml. 0·9% NaCl + 4·8 ml. M-$NaHCO_3$. (Breusch's (1943) medium). O_2.)

	Medium I				Medium II			
Substances added to medium	Nil	0·005 M-Citrate	0·01 M-$BaCl_2$	0·005 M-Citrate, 0·01 M-$BaCl_2$	Nil	0·005 M-Citrate	0·01 M-$BaCl_2$	0·005 M-Citrate, 0·01 M-$BaCl_2$
O_2 uptake (μl.)	1290	1790	830	1150	193	310	351	339
Citrate removed (μl.)	—	432	—	355	—	106	—	96

acceleration is feasible bearing in mind that a number of oxidative reactions in respiring cells compete with, and thus inhibit, each other; lactate, e.g., inhibits the oxidation of L-amino acids (see Krebs, 1935). It is feasible that barium ions inhibit a reaction which normally compete with, and thus slows down, the formation of citrate. This would result in an increased rate of citrate formation.

According to Breusch (1943*a*, *b*) the presence of bicarbonate in the medium, without CO_2 in the gas phase, also increases the yields of citrate from oxaloacetate. Table 13 shows that the oxygen uptake and citrate removal are greatly reduced in Breusch's medium. The increased yields of citrate in this case might thus be explained by the assumption that in this medium, which is unphysiologically alkaline, the formation of citrate is less inhibited than its removal.

DISCUSSION

Acetoacetate and the tricarboxylic acid cycle. The facts that the C_4-dicarboxylic acids promote the aerobic removal of acetoacetate and that acetoacetate increases the total yield of the acids of that cycle in the presence of oxaloacetate are in accordance with the conception, already safely established by tracer work, that acetoacetate is metabolized through the tricarboxylic cycle. So far there is no conclusive evidence suggesting alternative pathways of acetoacetate breakdown.

The main gap in the present knowledge of acetoacetate metabolism concerns the reactions leading from acetoacetate and oxaloacetate to *cis*aconitate. It is noteworthy that these reactions appear to require the presence of O_2, although the balance sheet of the reaction

$$\text{acetoacetate} + \text{oxaloacetate} \rightarrow \textit{cis}\text{aconitate}$$

does not represent an oxidation.

Our previous arguments (Krebs & Eggleston, 1945) against the hypothesis of Breusch (1943*a*, *b*) and of Wieland & Rosenthal (1943) were based on anaerobic experiments, where practically all the acetoacetate removed in the presence of oxaloacetate was accounted for as β-hydroxybutyrate. We pointed out that Breusch and Wieland & Rosenthal had supplied no evidence that under their experimental conditions acetoacetate was removed by a reaction other than reduction and that an alternative explanation was available for the increased yields of citrate. These arguments are now superseded by the tracer work of Buchanan *et al.* (1945) and Weinhouse *et al.* (1945, 1946).

Mechanism of condensation. The following considerations are offered as a contribution to the unsolved problem of the mechanism whereby oxaloacetate condenses with a second molecule to form the 6-carbon skeleton of the tricarboxylic acids. The tracer experiments have made it clear that citrate cannot be an intermediate because the isotopic carbon introduced as $CH_3.{}^{13}CO.CH_2.{}^{13}COOH$ was unsymmetrically distributed over the two carboxyl groups of α-ketoglutarate, the major part being in the γ-carboxyl. These facts are in accordance with the view that *cis*aconitate is the primary product of condensation provided that the reaction *cis*aconitate → *iso*citrate → α-ketoglutarate is very rapid in comparison with the reaction *cis*aconitate → citrate. Experimental evidence supporting this assumption has been presented previously (Krebs, 1943).

By analogy with well known organic chemical reactions a possible mechanism of formation of *cis*aconitate would be an aldol condensation of the type of the Perkin reaction or Knoevenagel reaction:

$$R.CHO + CH_3COONa \rightarrow R.CH{:}CH.COONa.$$

Applied to oxaloacetate and an acetate residue this type of condensation would yield a *cis*aconitic acid with the double bond in the 2:3 position to the carboxyl of the acetate residue. This would form an α-ketoglutarate whose carboxyl adjacent to the α-ketonic group is derived from the carboxyl of the acetate residue. The tracer experiments show that this is not the case. No double bond is formed as a result of the enzymic condensation. The double bond in *cis*aconitic acid, as already assumed by Wood *et al.* (1941), must be derived from that of the *enol*oxaloacetate (hydroxymaleic acid).

SUMMARY

1. The aerobic removal of acetoacetate by minced sheep heart and kidney cortex was investigated and a number of metabolites formed in the presence of acetoacetate were determined.

2. Manometric methods were developed for the determination of pyruvate in the presence of oxaloacetate and of oxaloacetate in the presence of pyruvate plus acetoacetate. In elaborating these methods it was noted that oxaloacetate gives variable yields of CO_2 with yeast carboxylase and that mixtures of acetoacetate and α-ketonic acids (pyruvate, α-ketoglutarate) behave towards Al ions like dicarboxylic β-ketonic acids, which suggests that these mixtures contain β-ketonic acids formed by aldol condensation.

3. In heart and kidney cortex some of the added acetoacetate was rapidly reduced to β-hydroxybutyrate even under aerobic conditions, whilst another fraction underwent oxidation. The rate of oxidation was increased by oxaloacetate and by fumarate. Data on the rates of reduction and oxidation of acetoacetate under varying conditions are given.

4. Nine metabolites (oxaloacetate, acetoacetate, pyruvate, β-hydroxybutyrate, succinate, fumarate,

L-malate, citrate, α-ketoglutarate) found in solutions as a result of the interaction between acetoacetate and oxaloacetate were determined in aerobic and anaerobic experiments. The most notable effect of oxygen on the yields of various substances was a higher yield of citrate. Calculations based on the yields suggest that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle, a conclusion now firmly established by the tracer work of Buchanan *et al.* and of Weinhouse *et al.*

5. Erythro-1:2-dihydroxybutyric acid was not metabolized by sheep kidney cortex whilst the threo form was slowly oxidized. In accordance with Weil-Malherbe's findings, the slowness of the oxidation excludes this substance as an intermediate in the removal of ketone bodies.

6. Measurements of the rates of α-ketoglutarate formation from various substrates and substrate combinations showed that the tricarboxylic acids yield α-ketoglutarate more rapidly than any other substrate. This supports the assumption that α-ketoglutarate arises via the tricarboxylic acids when formed in the presence of oxaloacetate and acetoacetate (or pyruvate). The rate of citrate formation via carboxylation of α-ketoglutarate (Ochoa, 1945) was relatively slow in sheep kidney cortex.

7. The sum of *cis*aconitate plus *iso*citrate was found to exceed 10·5% of the total tricarboxylic acids (maximum 49%) under suitable conditions. It is pointed out that this finding excludes the sequence (4) (p. 302) and supports the sequence (3) (p. 301), confirming conclusions earlier drawn from tracer work.

8. It was confirmed that barium (Wieland & Rosenthal, 1943) and bicarbonate (Breusch, 1943*a*, *b*) ions increase the yield of citrate from oxaloacetate and acetoacetate. Possible causes of this effect were investigated.

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The Isolation of Hydroxylysine Picrate from a Gelatin Hydrolysate

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Schryver, Buston & Mukherjee (1925) claimed the isolation, from isinglass hydrolysate, of an 'oxylysine' to which they attributed the formula $C_6H_{14}O_3N_2$; but they were unable, on repeated attempts, to prepare a crystalline picrate. Van Slyke & Hiller (1921) reported the presence of hitherto unknown material in the basic amino-acid fraction of hydrolyzed gelatin; this base was isolated by Van Slyke, Hiller, Dillon & MacFadyen (1938) who ascribed to it the formula of a hydroxylysine. After removal of arginine and histidine, these workers freed the 'lysine fraction' from monoamino-acids, and then removed the greater portion of the lysine by the addition of sufficient picric acid to combine with three quarters of the amino nitrogen present. A picrate of composition $C_6H_{14}O_3N_2 . C_6H_3O_7N_3$, which melted at 225° when heated slowly, was eventually isolated from the mother liquor. Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) and Van Slyke, Hiller & MacFadyen (1941)

have since endeavoured to establish the structure of hydroxylysine, and to determine its presence in other proteins by adaptation of the periodate oxidation reaction of Nicolet & Shinn (1939).

Martin & Synge (1941), by the application of an acetylation-benzoylation procedure to the lysine fractions of gelatin and isinglass hydrolysates, isolated in each case a small amount of a crystalline picrate which exploded at 226–227°. Although their analytical figures for formaldehyde and ammonia evolved on periodate treatment were low, this work is the only independent confirmation, by isolation, of the existence of the base first described by Van Slyke *et al.* (1938).

Additional evidence has been forthcoming from the periodate reaction besides that supplied by Martin & Synge. Using Vorländer's (1929) technique for determining the formaldehyde produced by oxidation of the 'lysine fraction' of isinglass, Beveridge & Lucas (1944) obtained a value from which they deduced the probable presence of some 0·36 % hydroxylysine. It was not apparent whether the compound estimated was that described by Schryver *et al.* (1925), or by Van Slyke *et al.* (1938), and accordingly they attempted the isolation advocated by the American workers, but failed to obtain any hydroxylysine picrate. More recently, Rees (1946) has carried out estimations by periodate oxidation which have confirmed the presence of a basic hydroxy amino-acid in gelatin and collagen, but not in any other proteins examined. Desnuelle & Antonin (1947) have criticized Van Slyke's figures based on the production of ammonia in the periodate reaction when carried out in the presence of excess glycine, and have preferred a colorimetric estimation of the formaldehyde simultaneously liberated. They found no evidence for the presence of hydroxylysine in casein, which contains, according to Van Slyke and his co-workers, 0·33 %. Neither Rees (1946) nor Desnuelle & Antonin (1947) attempted the isolation of any derivatives.

The isolation of a hydroxylysine picrate, which agrees in melting point and other physical properties with the compound isolated by Van Slyke and which affords formaldehyde and ammonia on periodate oxidation is described in this paper. The separation was effected by means of fractional recrystallization of the product derived from the 'lysine fraction' of a purified gelatin catholyte.

MATERIALS AND METHODS

Gelatin (1 kg.) known commercially as 'Spa Brand', kindly supplied by Messrs B. Young and Co. Ltd., of Bermondsey and containing 2 % ash, 15 % moisture and 13·82 % (uncorr.) nitrogen was hydrolyzed by boiling for 24 hr. with 2·5 l. of 30 % (v/v) H_2SO_4; after dilution, the H_2SO_4 was removed first to pH 2·5 and finally to pH 5·5 with $Ba(OH)_2.8H_2O$. The hydrolysate, together with washings of the precipitates, was then concentrated *in vacuo* to *c.* 1 l., and subjected to fractional electrodialysis in a three-compartment cell to remove all but traces of monoamino-acids from the catholyte. From this purified basic fraction, arginine and histidine were completely removed with flavianic acid, and excess flavianic acid with baryta. Finally the excess Ba^{++} was entirely removed with H_2SO_4 and ammonia by concentration *in vacuo*.

EXPERIMENTAL

Main picrate fraction

Preparation. The 'lysine fraction' referred to above (700 ml.) was precipitated with 45 g. (excess) of picric acid, affording a picrate which, after recrystallization from 1 l. of water, weighed 24 g. and exploded at 267°, the usually accepted explosion point for lysine picrate. Recrystallization of this picrate from water produced no change in explosion point.

Conversion to dihydrochloride. A portion (10 g.) of this twice recrystallized main picrate fraction was converted into the dihydrochloride (Cox, King & Berg, 1929). Although free from arginine, histidine and ammonia, the product melted at 192–193°, i.e. about 10° below the accepted melting point of lysine dihydrochloride and was also very hygroscopic.

Analysis of the dihydrochloride by HIO_4 *oxidation.* The dihydrochloride (0·1 g.), prepared as above, was dissolved in water (5 ml.) and HIO_4 (0·1 g. in 10 ml. of water) added. The oxidation was carried out in the manner described by Van Slyke *et al.* (1940). Subsequent treatment with Vorländer's reagent, 'dimedone' (5:5-dimethyl*cyclo*hexane-1:3-dione) precipitated an insoluble condensation product which was identified by its m.p. (185°) as methylene bis-dimedone. The melting point of this compound was not depressed on admixture with authentic material (m.p. 189°). Assuming the formaldehyde to have been derived from a hydroxylysine, the yield of dimedone derivative, 1·61 mg., corresponds to the presence of some 1·3 g. of the hydroxylysine salt/100 g. of dihydrochloride. (This figure must be regarded as minimal and the true content may well be higher.)

The suggestive analytical evidence obtained from the main picrate fraction warranted a search for greater quantities of the presumptive hydroxylysine in the main picrate mother liquors. Fractionation of these did, indeed, lead to the isolation of a picrate having the properties of that of hydroxylysine.

Fractionation of the main picrate mother liquors

The mother liquor and the washings (*c.* 1 l.) from the main crop of picrate were evaporated to 50 ml. affording, after one recrystallization, a second crop P_1 (1·9 g.) of explosion point 254°. Recrystallization of P_1 from water yielded 1·76 g. of P_2 (explosion point 259°). From the filtrate and washings, containing 0·14 g. of mixed picrates, was obtained, after evaporation to a few ml. and cooling to room temperature, a picrate P_3, paler yellow in colour than the picrate of lysine.

P_3, after filtration, washing with ice water and drying *in vacuo*, melted at *c.* 234°, with decomposition and bubbling but without explosion. Recrystallization from the minimum amount of water gave picrate P_4 of similar melting point and so the solvent was changed to 50 % (v/v) aqueous

ethanol. This solution was boiled with charcoal, filtered and evaporated to dryness to give P_5. The latter was taken up in boiling methanol containing a little water and allowed to crystallize at 5°. The picrate so formed melted with decomposition at 225°, and this melting point was unchanged on further recrystallization from aqueous ethanol.

Properties of the picrate isolated

The compound was finally isolated in a yield of some 80 mg. When heated at the rate of 1° every 3 sec., it showed the same m.p. (225°) as the hydroxylysine picrate isolated by Van Slyke *et al.* (1938). The change was fairly sharp, but, though decomposition took place at the same time, there was no explosion such as occurs with lysine picrate. The intermediate fractions, P_3, P_4, showed the typical decomposition of impure lysine picrate. The crystals of the new compound, which consisted of pale-yellow needles possessing a tendency to sheaf-like formation, appeared under the microscope very similar in shape to those of lysine picrate. Crystallization in the test tube took place rapidly from a supersaturated solution and a fluffy precipitate then formed. This dull, lemon-yellow precipitate was quite different in appearance from the characteristic, bright, golden-yellow colour associated with the picrate of lysine.

Oxidation by periodate. Samples of about 20 mg. each of the new picrate were treated with 0·5 M-HIO_4, and the resulting formaldehyde was precipitated as the dimedone derivative, washed and dried at 110° to constant weight. Duplicates agreed to within 2·4%, but the yield of methylene bis-dimedone varied with the conditions of condensation. It is now known, from the work of MacFadyen (1945), in which the dimedone-formaldehyde condensation was studied by means of the chromotropic acid reaction that, for 'complete' (98%) precipitation, up to 29 hr. at 39°, may be necessary if the condensation is carried out at pH 3·5, though the time required rapidly diminishes as the pH rises. At the time that these experiments were being carried out, however, it was not realized that the conditions of precipitation were so important. In all experiments, precipitation was carried out in a medium which was acid to methyl red and at room temperature. In one set of experiments, where the mixture was cooled immediately after the addition of dimedone and then allowed to stand in the refrigerator for 18 hr., the yield corresponded to only 62% of theory for hydroxylysine picrate; when the mixture was allowed to stand at room temperature for about 3 hr. before refrigeration, the yield rose to 81·8%.

Lack of material prevented the carrying out of further analyses. There seems little doubt, however, when the results of the periodate oxidation are considered in conjunction with the other properties of the picrate isolated from these mother liquors, that the base in question was hydroxylysine. The claims of Van Slyke *et al.* (1938) are thus confirmed.

DISCUSSION

It appeared impossible to purify the picrate mixture P_3, melting at *c.* 234°, by further recrystallization from water although, on changing the solvent, the heterogeneity of the substance became apparent and eventually hydroxylysine picrate of lower melting point was separated from the mixture. Thus the evidence strongly indicates that a solid solution separates from the aqueous system.

In this connexion, it may be pointed out that Van Slyke *et al.* (1941) found that diamino-acids are precipitated from mixtures by phosphotungstic acid partly as mixed salts rather than as individual substances each with its own independent solubility. These authors have shown further that in a mixture of lysine and hydroxylysine so precipitated the amount of hydroxylysine in the filtrate varies inversely to the ratio of lysine/hydroxylysine in the precipitate. The observations contained in this paper on the picrates are in good agreement with the above, and are of some practical importance in indicating that the isolation of hydroxylysine, whether by fractional precipitation with picric acid or by fractional recrystallization, depends ultimately on the solubilities of the picrates in water and in one another.

In view of this evidence, it would seem unlikely that hydroxylysine of 100% purity could be obtained by ordinary purification procedures. Gale (1947) has recently tested a decarboxylase, claimed to be specific for L-lysine, on samples of hydroxylysine received from Van Slyke. These samples were slowly, but incompletely, attacked. Zittle & Eldred (1944), using the same organism on zein hydrolysates, have shown that hydroxylysine cannot be decarboxylated to any appreciable extent. Although the amounts of lysine and hydroxylysine in zein are somewhat uncertain, it is possible that the results of Gale (1947) are due, in part, to contamination of the hydroxylysine with lysine.

SUMMARY

1. Hydroxylysine, the amino-acid prepared from gelatin by Van Slyke and co-workers has been independently isolated by fractional recrystallization of its picrate, from a sample of crude lysine picrate. Some of the properties of hydroxylysine picrate have been described.

2. The difficulties encountered in the separation of hydroxylysine picrate from that of lysine by fractional crystallization are stressed, and evidence is produced indicating that even the best samples of hydroxylysine so far prepared are not entirely free of lysine.

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The Reaction of Nucleic Acids with Mustard Gas

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The delayed healing of lesions caused by mustard gas (*H*) provoked speculation that the cell nuclei are especially concerned, and confirmation of this view has accumulated from various sources. Auerbach & Robson (1942, 1946) showed that the exposure of *Drosophila melanogaster* to *H* in sublethal doses caused reduced fertility and induced visible mutations in succeeding generations. Robson (1943) observed that pollen grains of *Tradescantia* after treatment with *H*, showed widespread disorganization of chromosomes, and Fell & Allsopp (1943) noted similar behaviour in the cells of *H*-treated mouse skin. Koller (1943) records that during the 48 hr. following a short (30 sec.) exposure of *Tradescantia* pollen grains to concentrated *H* vapour, chromosome breakage, structural changes including new rearrangements, and adherences of adjacent chromosomes were induced at various loci in the chromosomes. He observed that the separation of the chromosomes at anaphase and telophase of mitosis was prevented by these attachments. These results focused attention on the action of *H* on nucleoproteins, and Berenblum (1940 *a*, *b*) and Berenblum & Schoental (1947) found that aqueous extracts of skin or solutions of thymus nucleoprotein underwent irreversible precipitation when treated with *H*, the precipitate having a higher sulphur content than the original nucleoprotein.

The action of *H* on proteins has been studied by many investigators, e.g. Berenblum & Wormall (1939); for reviews see Rydon (1943 *a*, *b*). The purpose of the present investigation was therefore to investigate the reaction of *H* with nucleic acids and their components.

EXPERIMENTAL

Choice of nucleic acids for investigation. The ribose nucleic acid of yeast (YNA) and the deoxypentose nucleic acid of calf thymus gland (TNA) were employed in this investigation because of their availability, and because more is known of their structures than of those of nucleic acids from other sources.

Preparation of materials. The samples of yeast ribonucleic acid were obtained commercially and were deproteinized by a combination of the method described by Sevag, Lackmann & Smolens (1938) and the purification process employed by Fletcher, Gulland, Jordan & Dibben (1944). Yeast ribonucleic acid (20 g.), stirred vigorously into water (250 ml.), was brought into solution at pH 7 by the addition of N-NaOH (about 48 ml.). This solution was shaken vigorously with the following volumes of a mixture (4 : 1) of chloroform and amyl alcohol: 200 ml. four times, 100 ml. ten times. After each shaking, the mixture was centrifuged and the aqueous layer containing the nucleic acid was separated from the chloroform layer which contained the protein as a stable gel. When gel formation no longer occurred after shaking, the solution of protein-free nucleic acid was slowly added to a stirred mixture of ethanol (1180 ml.) and concentrated HCl (6·6 ml.). The precipitated acid (12 g.) was centrifuged and washed three times with 66% aqueous ethanol when it was found to be free from chloride ions; it was then washed successively, once with 80 and 100% ethanol, finally with ether, and dried in a vacuum over P_2O_5-KOH at room temperature. Analyses of various samples are recorded in Table 2.

Deaminated yeast ribonucleic acid (DYNA) was prepared from YNA as described by Fletcher, Gulland, Jordan & Dibben (1944). The samples of the sodium salt of deoxypentose nucleic acid of low molecular weight were obtained commercially and deproteinized by the procedure described above. The sample of high molecular weight had

been prepared from calf thymus glands by Gulland, Jordan & Threlfall (1947). Analyses are given in Table 2. Guanylic acid in the form of its sodium salt was prepared by alkaline hydrolysis of YNA. The pure *H* used was provided by the Chemical Defence Research Department, Porton.

paper. (Before these tests were made, the samples were thoroughly washed with ethanol and ether.) The absence of free *H* in the products was confirmed by the consistent failure to detect the presence of chlorine as a component by means of qualitative tests.

Table 1. *Conditions of reaction of H with nucleic acids*

Reference no. of nucleic acid	Wt. of nucleic acid (g.)	Wt. of *H* (g.)	Mol. of *H*/tetra-nucleo-tide	Vol. of solution (ml.)	Time of stirring (hr.)	Reference no. of A product	Yield of A product (g.)	Reference no. of B product	Yield of B product (g.)	Yield of A / Yield of B
YNA:										
Y3/20	1·50	0·75	4·1	8·0	10·0	HY3/22A	1·00	HY3/22B	0·55	1·82
Y4/26	4·00	5·00	10·2	30·0	6·0	HY4/30A	3·60	HY4/30B	0·30	12·00
Y5/42	3·00	1·90	5·1	20·0	10·0	HY5/45A	1·40	HY5/45B	0·90	1·56
Y5/42	3·00	1·90	5·1	40·0	10·0	HY6/46A	1·15	HY6/46B	0·85	1·35
Y5/42	3·00	1·90	5·1	60·0	10·0	HY7/47A	1·50	HY7/47B	0·75	2·00
Y6/48	3·00	1·90	5·1	20·0	3·0	HY8/49A	0·10	HY8/49B	3·05	0·03
Y6/48	3·00	1·90	5·1	20·0	6·0	HY9/50A	0·50	HY9/50B	2·55	0·20
DYNA:										
DY2	0·50	0·25	4·1	8·0	22·0	HDY1/25A	0·15	HDY1/25B	0·25	0·60
DY8	6·80	8·00	9·6	48·0	7·0	HDY2/34A	2·18	HDY2/34B	4·10	0·53
HDY2/34B	2·00	3·00	13·7	20·0	10·0	HHDY3/43A	1·00	HHDY3/43B	0·10	10·00
TNA:										
T1/19*	0·75	0·70	7·3	8·0	10·0	HT1/27A	0·75	HT1/27B	0·02	37·5
T2/31*	1·50	1·50	7·3	23·0	11·0	HT2/36A	1·15	HT2/36B	0·35	3·3
T5†	2·00	1·30	5·8	450·0	9·0	HT3/53A	0·01	HT3/53B	1·60	0·006

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Degraded low mol. wt. samples. † High mol. wt. sample (tetrasodium salt).

Table 2. *Analytical data for H-treated nucleic acids*

Nucleic acid			A product					B product			
Reference no.	N (%)	P (%)	Reference no.	S (%)	N (%)	P (%)	Na (%)	Reference no.	S (%)	N (%)	P (%)
YNA:											
Y3/20	16·7	9·1	HY3/22A	4·4	14·3	7·6	—	HY3/22B	4·3	14·9	8·2
Y4/26	16·5	9·4	HY4/30A	3·1	14·5	8·1	3·2	HY4/30B	2·8	14·4	8·6
Y5/42	15·9	9·2	HY5/45A	3·5	13·6	7·7	—	HY5/45B	3·8	13·5	8·3
Y5/42	15·9	9·2	HY6/46A	4·3	13·2	7·5	—	HY6/46B	4·7	13·5	8·2
Y5/42	15·9	9·2	HY7/47A	4·9	13·4	7·6	—	HY7/47B	5·2	13·3	7·9
Y6/48	15·6	9·2	HY8/49A	1·6	14·0	7·7	—	HY8/49B	1·5	15·0	8·8
Y6/48	15·6	9·2	HY9/50A	3·0	14·0	7·6	—	HY9/50B	2·8	14·7	8·4
DYNA:											
DY2	11·7	7·6	HDY1/25A	5·0	—	—	—	HDY1/25B	5·1	11·2	7·4
DY8	—	—	HDY2/34A	4·3	10·6	7·2	3·4	HDY2/34B	4·6	10·4	7·9
HDY2/34B	10·4	7·9	HHDY3/43A	7·9	9·2	6·7	—	HHDY3/43B	6·7	8·3	7·5
TNA:											
T1/19*	—	—	HT1/27A	3·4	13·9	8·6	1·0	HT1/27B	—	—	—
T2/31*	16·4	9·5	HT2/36A	3·7	13·3	8·2	3·4	HT2/36B	3·5	12·9	8·9
T5†	15·3	9·3	HT3/53A	—	—	—	—	HT3/53B	4·9	13·3	8·6

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Degraded low mol. wt. samples. † High mol. wt. sample (tetrasodium salt).

Criteria of reaction of H with nucleic acids. The criteria of positive chemical reaction between *H* and nucleic acids were as follows: (i) the qualitative demonstration of the presence of sulphur in the products, followed later by detailed analytical investigation; (ii) the demonstration of the absence of uncombined *H* in the products by prolonged passage of air from the sample through S.D. (1942) reagent

The reaction of H with nucleic acids. No interaction could be detected between YNA and *H* as vapour or dissolved in liquid paraffin. When, however, both were dissolved in aqueous ethanol at pH 7, a reaction occurred. The solubility of *H* in aqueous ethanol increases with the concentration of alcohol, whereas that of nucleic acids decreases. The highest concentration of ethanol which could be employed without

the precipitation of nucleic acid was 35%, and this concentration was employed throughout the experiments described below; the presence of ethanol, moreover, considerably reduces the hydrolysis of *H* (Mohler & Hartnagel, 1941; Ogston, 1941) which is a serious competing reaction. When the reaction was carried out in aqueous media with *H* in suspension, the reaction between *H* and nucleic acid was found to be almost negligible.

The results of a series of experiments made with different proportions of *H* and YNA are summarized in Tables 1 and 2. Two products, designated A and B respectively, were always formed, but in various proportions depending on the conditions of the experiment. The A compounds separated from the reaction mixture at pH 7 during the course of the experiment, whereas the B compounds were precipitated by the addition of a mixture of HCl and ethanol after removal of the A compounds at the conclusion of the preparation.

The following description is typical of the experiments which were carried out, and illustrates the procedure. Yeast ribonucleic acid (Y 5/42; 3 g.) was suspended in water and brought into solution at pH 7 by the addition of approximately N-NaOH (about 7 ml.), the final volume being 13 ml. Ethanol (7 ml.) was added, then *H* (1·9 g.), and the mixture was stirred mechanically for 10 hr., the reaction being frequently adjusted to pH 7 by the addition of NaOH. The solid, a sodium salt which had separated, was centrifuged, washed several times with dry ethanol, twice with ether, and dried *in vacuo* over P_2O_5-KOH. Yield 1·4 g. (HY 5/45 A).

The supernatant liquid was then added slowly to a stirred mixture of ethanol (110 ml.) and concentrated HCl (0·6 ml.). The precipitated solid was centrifuged, washed with ethanol and then with ether, and dried *in vacuo* as before. Yield 0·95 g. (HY 5/45 B).

The reactions of *H* with DYNA and with TNA were carried out by a similar procedure. The results are summarized in Tables 1 and 2.

The reaction of H with guanylic acid. The method described above for the reaction of *H* with nucleic acids gave only poor yields when applied to the reaction between *H* and guanylic acid and the following modified procedure, which gave a satisfactory yield, was developed. Sodium guanylate (3 g.) was dissolved in hot water (30 ml.), the solution cooled and added to a solution of *H* (11·8 g., 10 mol. ratio) in ether (15 ml.). The mixture was stirred for 72 hr. and kept neutral by periodic additions of NaOH. *H*-guanylic acid was precipitated with ethanol-ether (5 : 1) mixture (450 ml.). The gummy product was centrifuged and washed four times with ethanol and twice with ether. It was redissolved in water (50 ml.) and the *H*-guanylic acid reprecipitated as a solid by the addition of ethanol and ether; a further reprecipitation was necessary to free the product from NaCl. The final separation of the product by centrifuging was exceedingly difficult owing to its colloidal nature. The *H*-guanylic acid (2·1 g.) was dried *in vacuo* over P_2O_5.

Analytical methods. Nitrogen analyses of untreated and *H*-treated materials were carried out by a micro-Kjeldahl technique (Claudatus, 1939) modified by Still (unpublished). Phosphorus contents were determined by a modification (Still, unpublished) of the micro-method of Embden (1921).

Estimations of the sulphur content of *H*-nucleic acids were carried out by the adaptation by Niederl & Niederl (1938) of the Pregl combustion method. The accuracy of this method depends upon the presence of insufficient metal (e.g. sodium) to retain the S present as metallic sulphate. The *H*-nucleic acids conform to this requirement since the P : S ratio is relatively high. After the analysis, the phosphorus remained in the boat as sodium metaphosphate, which was in each case shown qualitatively to be free from sulphate. In some cases, estimations of sulphur were also made by the micro-Carius method; these results confirmed those obtained by the more rapid Pregl method.

The results of analysis for sulphur in *H*-guanylic acid were low and inconsistent by both the Pregl and Carius methods. In the Pregl method, sulphate could be detected in the boat, and the procedure was modified. The residue was extracted with very dilute HCl, the solution filtered and added to the main bulk of sulphate-containing solution; estimations of this sulphate as $BaSO_4$ then gave consistent results.

Sodium analyses were at first carried out by the method of Hoffmann & Osgood (1938). Later, however, a gravimetric method was used in which the sample was wet-ashed with concentrated HNO_3, phosphate removed as barium phosphate, and sodium weighed as Na_2SO_4.

Electrometric titrations were carried out by the method used by Fletcher, Gulland & Jordan (1944), corrections being applied as described by Jordan & Taylor (1946).

Viscosity measurements on *H*-TNA were carried out using Frampton's (1939) modification of the Ostwald viscometer.

RESULTS

Properties of H compounds

The A products from YNA were pale buff, amorphous solids, insoluble in water but dissolving near pH 10. The B products from YNA were cream-coloured solids, soluble in water above pH 3–4, but precipitated in more acid solutions.

The A compounds from DYNA were very similar in behaviour to those from YNA, being insoluble at a pH below 10. The B compounds were soluble in water and were not precipitated at pH 2·5; this solubility in acids is characteristic of DYNA (Fletcher, Gulland, Jordan & Dibben, 1944).

The A and B compounds from TNA resembled their analogues prepared from YNA in most respects, but exhibited at least one important difference in that their solutions were more viscous than those of the original TNA at corresponding concentrations. Aqueous solutions at pH 11–12 of the A compounds prepared from the low molecular weight samples T 1/19 and T 2/31 were far more viscous than the original nucleic acids, but quantitative determinations were not carried out. The viscosities of aqueous solutions of the sample T 5 of high molecular weight and of the B compound (HT 3/53 B) prepared from it are given for various pressures in Fig. 1. Solutions of HT 3/53 B exhibited critical pressures, in magnitude largely dependent on concentration; at pressures below the critical, the solutions formed a gel and did not flow. Critical

pressures have not been determined for T5 (Creeth, Gulland & Jordan, 1947), but if they exist are considerably lower than those of the B compound.

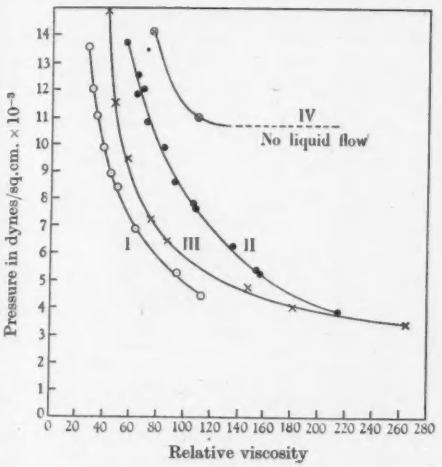

Fig. 1. The viscosities of aqueous solutions of calf thymus deoxypentose nucleic acid (T5) and its *H* product (HT3/53B). T5: curve I, ⊙, 0·21% solution; curve II, ●, 0·31% solution. HT3/53B: curve III, ×, 0·21% solution; curve IV, ⊗, 0·31% solution.

In air, these *H* compounds emitted a peculiar odour which was notably increased on treatment with acid and alkali. This odour was reminiscent of the thio-ethers and very similar to that obtained by the reaction of *H* with ethanolic alkali, known to give divinyl sulphide. In order to investigate the degree of the decomposition of the *H* compounds caused by the presumed evolution of divinyl sulphide, one sample was analyzed for sulphur immediately after preparation and again after storage for 5 weeks in a loosely corked tube at room temperature. The sulphur contents were 4·35 and 4·30%, respectively. The difference between the two figures is within experimental error and hence no extensive decomposition had occurred.

The identification of groups reacting with H

The electrometric titration of nucleic acids has proved to be a useful method of investigating the structures of nucleic acids, and in particular the number and type of acidic and basic groups present (Fletcher, Gulland & Jordan, 1944; Gulland, Jordan & Taylor, 1947). The titration curves of the *H*-treated materials have therefore been determined to compare the number of titratable groups present with that of the original nucleic acids. In using this method for the *H*-nucleic acids, however, it is appreciated that titration may produce fission of the *H* residues from the nucleic acids, especially in strongly acid and alkaline solutions, and the results obtained may therefore be slightly in error. Throughout, the calculations of numbers of reacting groups are based for convenience on the provisional assumption, sufficiently accurate for present purposes, that the nucleic acids are composed of statistical tetranucleotides (Gulland, Barker & Jordan, 1945; Gulland, 1947).

In order to interpret the data obtained by elementary analysis and by electrometric titration of *H* compounds, it is necessary to estimate for each sample the formula weight (M) of a statistical *H* tetranucleotide. This was obtained in each case from the sulphur content, assuming that (i) the number of P atoms/statistical tetranucleotide was the same as in the parent substance, and (ii) the reaction consisted in the replacement of hydrogen atoms by $—CH_2.CH_2.S.CH_2.CH_2.OH$ or $(—CH_2.CH_2)_2S$ groups, the formula weight thus increasing by 104 in the first and 86 in the second case for each sulphur atom introduced.

In the case of substitution by

$$—CH_2.CH_2.S.CH_2.CH_2.OH,$$

if x such residues combine with each statistical tetranucleotide of the parent substance having formula weight m, and if the sulphur content of the product be s% then $s = 3200x/(m+104x)$ and hence $x = sm/(3200-104s)$ and $M = m+104x$. Similarly, in the case of substitution by $(—CH_2.CH_2)_2S$ groups, $x = sm/(3200-86s)$ and $M = m+86x$.

The calculation of m presents no difficulty in the case of thymus deoxyribonucleic acid for which it has the value 1235, but in the case of yeast ribonucleic acid and its deamination product, allowance must be made for the deficiency in phosphorus below the theoretical value. Thus, if the percentage of phosphorus be p, m is now the formula weight of the hypothetical tetranucleotide corrected for the deficiency of phosphorus, and δ the atom deficiency of phosphorus/tetranucleotide, then the number of atoms of phosphorus/tetranucleotide is $4-\delta = mp/3100$. Since the loss of a phosphoryl group involves a decrease of 80 in the formula weight of the hypothetical tetranucleotide then $m = 1285-80\delta$, and combining with the above equation $m = 299{,}150/(310-8p)$.

The calculation of M for all *H* compounds of nucleic acid is thus possible and, using an analogous method, the formula weight of the *H* compound of guanylic acid can also be obtained.

The A products of *H*-TNA, *H*-YNA and *H*-DYNA were not sufficiently soluble in aqueous solutions to permit their examination by electrometric titration. The B products, however, were readily soluble, and the results of titrations performed with these compounds and *H*-guanylic acid are given in Figs. 2, 3, 4 and 5, together with the titration curves of the original substances. It was found to be immaterial whether glass or hydrogen electrodes were used,

since poisoning of the latter did not occur. Table 3 summarizes the number of titratable groups potentially capable of reacting with *H*, the number of such groups remaining unsubstituted after treatment with *H*, and (by difference) the number of groups blocked by *H*. It will be seen that in every case a reaction with *H* has occurred.

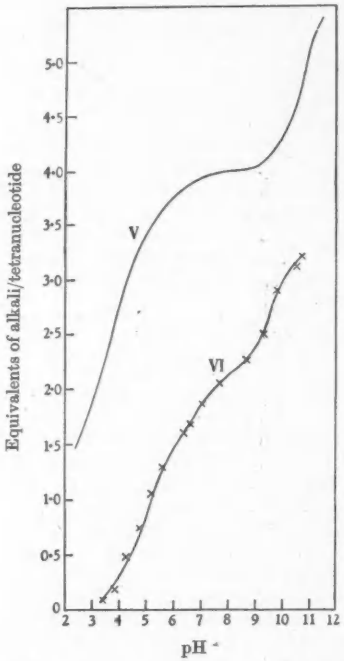

Fig. 2. The electrometric titration of calf thymus deoxypentose nucleic acid (T5), curve V (Gulland, Jordan & Taylor, 1947) and its *H* product (HT3/53B), curve VI, ×.

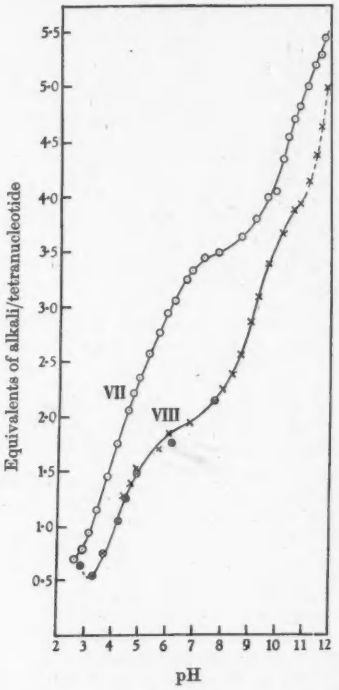

Fig. 3. The electrometric titration of yeast ribonucleic acid (Y5/42), curve VII, ⊙, and its *H* product (HY7/47B), curve VIII, ×⊗.

Table 3. *Numbers of titratable groups/tetranucleotide present in nucleic acids and numbers of groups titratable or blocked in their H compounds*

	Primary phosphoryl	Secondary phosphoryl	Amino	Purine-pyrimidine hydroxyl	Total groups blocked	$(-CH_2-CH_2)_2S$ residues*	$HOCH_2.CH_2$ S CH_2-CH_2 residues*
YNA, Y5/42	2·8	0·8	2·7–3·0	2·0	—	—	—
H-YNA, HY7/47B	1·6	0·4	≮1·2	1·8	—	—	—
Groups blocked	1·2	0·4	0–1·8	0·2	1·8–3·6	2·4	2·5
DYNA, DY8	2·5	1·2†	0	3·0	—	—	—
H-DYNA, HDY2/34B	1·2	0·6†	0	3·0	—	—	—
Groups blocked	1·3	0·6†	0	0	1·9	2·0	2·1
TNA, T5	4·0	0	3·0	2·0	—	—	—
H-TNA, HT3/53B	1·4	0·7‡	≮1·4	1·0	—	—	—
Groups blocked	2·6	(−0·7)	0–1·6	1·0	3·6–5·2	2·2	2·2
Guanylic acid	1·0	1·0	1·0	1·0	—	—	—
H-guanylic acid HG4/71	0·8	0·55	0·1	0·5	—	—	—
Groups blocked	0·2	0·45	0·9	0·5	2·05	1·85	2·03

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Calculated from sulphur analyses; see text. † Includes hydroxyl of xanthine. ‡ See text.

The titration curve of the B product from deoxypentose nucleic acid (T5) of high molecular weight is compared in Fig. 2 with that of a sample of the parent acid which has been treated with mineral acid; this procedure was adopted because the curve for the acid-treated substance differs slightly from that of the original (Gulland, Jordan & Taylor, 1947), and because the B compound was obtained by pre-

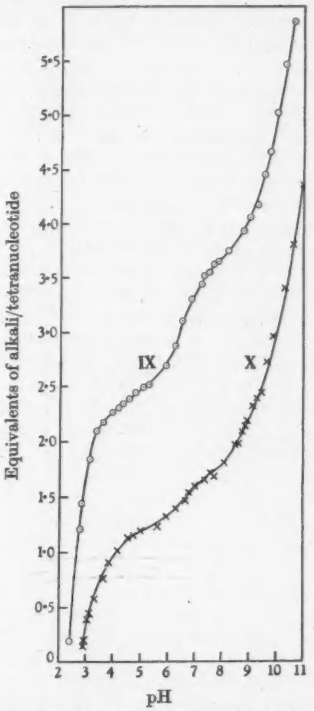

Fig. 4. The electrometric titration of deaminated yeast ribonucleic (DY8), curve IX, ⊙, and its *H* product (HDY2/34B), curve X, ×.

cipitation with mineral acid. The identity of the 0·7 equivalents of a group titrating between pH 6·0 and 8·4 is obscure; the only likely group titrating in this range is a secondary phosphoryl group, but the dissociation constant of the liberated group ($pK'_a = 7·2$) is different from that of the secondary phosphoryl group in thymic acid ($pK'_a = 6·4$) (Gulland, Jordan & Taylor, 1947) with which it may suitably be compared.

The dissociation constant of the purine-pyrimidine hydroxyl groups of yeast ribonucleic acid is changed from $pK'_a = 10·5$ to 9·4 by interaction of the acid with *H*. Such changes are not yet fully understood and have been discussed elsewhere (Fletcher, Gulland & Jordan, 1944; Gulland, Jordan & Taylor, 1947).

For the purpose of calculating the number of purine hydroxyl groups in *H*-guanylic acid, it is assumed that the dissociation constant of these is the same ($pK'_a = 9·5$) as that of guanylic acid; owing to the extensive removal of *H* residues by hydrolysis above pH 10, it was not possible to determine the complete titration curve.

The carbohydrate hydroxyl groups do not titrate below pH 12, and hence it was not possible to determine the extent, if any, of their reaction with *H* by electrometric titration.

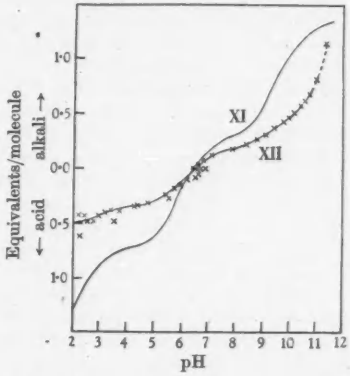

Fig. 5. The electrometric titration of guanylic acid, curve XI (theoretical curve), and its *H* product (HG4/71), curve XII, ×.

It is not possible to determine by titration alone the extent of the reaction of *H* with the primary phosphoryl group of guanylic acid. This can be calculated, however, on the assumption that no phosphorus is lost during the reaction. Analysis of the *H*-guanylic acid shows that the atom ratio P : Na is 1 : 1·2; thus there are in all 1·2 primary and secondary phosphoryl groups neutralized at pH 6·4 which is the pH of the *H*-guanylic acid solution in water. On titration with alkali (curve XII, Fig. 5) a further 0·15 equivalent of a secondary phosphoryl group is titrated. The total number of secondary and primary phosphoryl groups which have not reacted with *H* is thus 1·35. Since the titration shows that of these, 0·55 equivalent is due to secondary phosphoryl dissociation, it follows that 0·8 equivalent of a primary phosphoryl group has not reacted with *H* and hence 0·2 group has reacted. The total number of titratable groups blocked is 2·05.

DISCUSSION

Nucleic acids contain various groups which might be expected under suitable conditions to react with *H*. Assuming the generally accepted polynucleotide structure of the acids, the number and nature of these groups are summarized in Table 3, which is

based on the polymerization of a statistical tetranucleotide.

Of the various types of reaction into which *H* can enter, that in which the chlorine atoms are replaced by other substituents seems the most probable in the present circumstances, i.e. of the Hughes-Ingold S_N1 type (Watson, 1938; Hughes, 1941). *H* enters into such reactions with amino groups (Clarke, 1912; Helfrich & Reid, 1920; Cashmore & McCombie, 1923; Lawson & Reid, 1925; Ford-Moore, 1940; Boursnell, Francis & Wormall, 1946; Moore, Stein & Fruton, 1946), phenolic anions (Helfrich & Reid, 1920; Ford-Moore, 1940) and carboxylic anions (Doering, Linstead & Ball, 1942; Moore *et al.* 1946), but not with the undissociated carboxyl group (Cohen & Harris, 1942). Inspection of the titration curves of nucleic acids and nucleotides shows that in solution at pH 7 the phosphoryl groups are in the ionic form and the amino groups undissociated, and are thus likely to react with *H*. The purine-pyrimidine hydroxyls ($-N=\overset{|}{C}-OH$) titrate over the range pH 8·0–11·5, being thus similar to phenols in character, and would not be expected to react at pH 7. In DYNA, one hydroxyl group of xanthine is partly dissociated at pH 7 and should therefore react with *H*, whereas the other hydroxyl does not titrate at reactions more acid than pH 12. The sugar hydroxyls have $pK'_a = 13$ and would therefore not be expected to react at pH 7.

The number of titratable groups blocked by *H* is compared with the number of *H* residues calculated from the sulphur analyses (Table 3). It will be seen that in general the results agree with those predicted on theoretical grounds, although the numbers of groups reacting could not always be precisely determined. The only marked divergence is the reaction of the purine-pyrimidine hydroxyl in TNA and guanylic acid; this was unexpected but no satisfactory explanation can be offered.

In the B product from DYNA (HDY 2/34 B) and in the *H* compound from guanylic acid (HG 4/71), it is evident that within experimental error the number of *H* residues is equal to the total number of titratable groups blocked. It is therefore concluded that each titratable group blocked is substituted by one *H* residue. It seems probable, although not certain, that this residue is in the form

$$-CH_2.CH_2.S.CH_2.CH_2.OH,$$

since none of the products contained chlorine. It is considered unlikely that *H* has attacked simultaneously a titratable group and a non-titratable hydroxyl, because these latter would not be expected to undergo alkylation at pH 7. Two criticisms of this conclusion should be mentioned, although neither is regarded as likely to be valid. First, it is possible that half of the reacting *H* molecules have cross-linked non-titratable groups and the other half entered into cross linkages with titratable groups; such circumstances, although in agreement with the analytical results, are considered not only to be statistically improbable but also unlikely to occur at pH 7. Secondly, there is the possibility that the reactions with *H* are more complex in character and resemble the formation and decomposition of sulphonium salts known to occur between *H* and its substituted derivatives (Bell, Bennett & Hock, 1927). This reaction mechanism is considered to be most improbable since the formation of sulphonium salts is exceedingly slow at room temperature, and also since no 'sulphonium' dissociation was detected by electrometric titration. It is also possible that *H* has reacted with the amino groups to form 1:4-thiazan derivatives, a type of reaction observed under more vigorous conditions by Clarke (1912), Helfrich & Reid (1920), Lawson & Reid (1925) and Boursnell *et al.* (1946). Our data do not permit a decision as to the nature of the reaction undergone by the amino groups, and it is noteworthy that Moore *et al.* (1946) were unable to establish whether the amino groups of amino-acids and peptides formed secondary amines or thiazans in their reaction with *H*.

The precise figure for the total number of groups blocked in *H*-YNA (HY 7/47 B) could not be ascertained by electrometric titration, but since the number of the primary and secondary phosphoryl and purine-pyrimidine hydroxyl groups blocked is similar to that in *H*-DYNA (HDY 2/34 B), it appears unlikely that any extensive cross linkage has occurred.

In the B product from TNA, on the other hand, it is clear that the number of blocked titratable groups is greater than the number of *H* residues in the molecule, indicating that *H* molecules have reacted with two titratable groups, either in the same molecule or by a process of cross linking between polynucleotide chains. The comparison of viscosities of solutions of TNA before and after treatment with *H* provides additional evidence in favour of some measure of intermolecular cross linkage in view of the increased viscosity of the treated sample.

The calculated analytical and titration data for complete and no cross linking by *H* are compared with the observed data for *H*-guanylic acid in Table 4. The evidence confirms the theory that each titratable group is attached to one *H* residue and that there is little or no cross linking. Such a comparison is not satisfactory in the case of the nucleic acids as the calculated values for complete cross linking and no cross linking are too close together to permit distinction by analysis and titration.

The formation of two series of products, not one, by the interaction of nucleic acids with *H* at pH 7

must now be considered. The salient facts are as follows: (i) both A and B products were free from chlorine and contained unsubstituted phosphoryl groups, the members of the A series being sparingly soluble sodium salts (Table 2), dissolving only at reactions more alkaline than pH 10, whereas the B products were precipitated by mineral acid from solutions of their sodium salts; (ii) the ratio 'yield of A/yield of B' in any given instance increased with longer duration of reaction and higher molecular ratios of *H*/tetranucleotide, but was practically independent of the volume of the reaction mixture (Table 1); (iii) there was no marked or constant difference in the sulphur contents of the A and B products (Table 2); (iv) further treatment of a B compound gave rise to a new pair of A and B products, both containing considerably more sulphur (Table 2, HHDY 3/43 A/B). It is therefore clear that related members of the A and B series were not identical, and that the B products were not direct precursors of the A products formed simultaneously and having approximately the same sulphur content.

Table 4. *Comparison of observed and calculated data for H-guanylic acid (HG 4/71)*

Analysis	Calculated for no cross linkage	Calculated for complete cross linkage	Observed
No. of groups blocked/mol.	2·03	1·85	2·05
(%)			
C	36·2	38·1	37·0
H	4·9	4·9	4·7
N	11·7	12·8	11·8
P	5·2	5·7	5·3
S	10·8	10·8	10·8
Na	4·6	5·0	4·7

Two explanations of these differences may be mentioned, and these could operate singly or simultaneously. It is possible that the A products have been produced by the cross linking by *H* of lyophilic groups on the same polynucleotide chain; thus two solubility-conferring groups would be rendered inactive for each atom of sulphur introduced. Alternatively, the A products may have a much higher molecular weight than the B products as a result of the cross linking occurring between different polynucleotide chains. The B products are considered to be mainly the monosubstituted derivatives of thiodiglycol (see above). These hypotheses are in conformity with the possibility that B compounds on further treatment with *H* can produce either B compounds of higher sulphur content or by cross linking, A compounds of higher sulphur content (see (ii) and (iv) above).

There is thus ample evidence that at the pH values pertaining in tissues both deoxypentose nucleic acid of calf thymus and yeast ribonucleic acid will react with *H*. It is to be emphasized that it is not known whether the structure of pentose and deoxypentose nucleic acids of all tissues are the same as or different from those of YNA and TNA (Gulland, 1944, 1947; Gulland *et al.* 1945; Davidson & Waymouth, 1944–5), but there is no evidence to suggest that the reactive groups present in nucleic acids from other sources are not similar to or identical with those of YNA and TNA. The biological implications of the results are clear. It is generally agreed that deoxypentose and pentose nucleic acids are constituents of all nuclei, and are involved in the mechanism of mitosis. The attack of cell nucleic acids by *H* would interfere with this mechanism and hence with the proliferation of tissues injured by *H*. The present investigation thus provides a chemical parallel to and possibly an explanation of the cytological observations of Koller (1942), Robson (1943) and Fell & Allsopp (1943).

SUMMARY

1. It has been found possible to induce reaction between nucleic acids, guanylic acid and mustard gas (*H*) under mild conditions of pH and temperature.

2. The products of the reaction between nucleic acids and mustard gas have been isolated and analyzed. They are found to contain sulphur but not chlorine.

3. Electrometric titration of the *H* compounds shows that the primary and secondary phosphoryl groups, and the amino groups have in every case, when present, reacted with *H*. The purine-pyrimidine hydroxyl groups have been found to react only in certain cases.

4. The viscosity of solutions of the *H* compound of a high molecular weight sample of deoxypentose nucleic acid was found to be much greater than that of the original acid.

5. Possible structures of the *H* compounds are discussed, and the significance of the results considered in relation to the delayed healing of mustard gas lesions.

It is a pleasure to record our thanks to the Chief Scientist, Ministry of Supply, for permission to publish these results; to Mr J. E. Still for performing a number of the microanalyses; to Mr J. M. Creeth for the determinations of viscosities; to Mr W. E. Fletcher for the titration curve of DY 8; to Imperial Chemical Industries Ltd. for the loan of apparatus; and to the British Empire Cancer Campaign for a maintenance grant to one of us (D. T. E.) during a short period at the end of the work here recorded.

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The Component Acids of Herring Visceral Fat

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The component acids of North Sea herring flesh fats laid down at different seasons, including those of intensive feeding when much fat is deposited, and those of relative inanition when the body fat content falls to a low level, have been discussed by Lovern (1938). Similar data for the flesh fat of Icelandic herring have been given by Bjarnason & Meara (1944). The component acids of fats from other parts of the herring have not been studied in detail hitherto. Some time ago Mr J. R. Bruce, of the Marine Biological Station, Port Erin, Isle of Man, sent to us a quantity of oil extracted from the viscera of herrings taken locally, and we have now had an opportunity to examine the nature of the component acids of the visceral fat, which displays interesting differences from the characteristic composition of herring flesh fats.

METHODS

The visceral oil studied was from herrings caught off the south-western coast of the Isle of Man during the first week of August, 1942. The herrings had been gutted by hand in a local factory prior to conversion into kippers. The offal would have contained intestines, stomach, liver, mesentery, etc., and also the milt or roe, together with a very small number of heads and of broken fish (these forming, however, only a minute percentage of the whole). It was rendered by boiling with water in the Port Erin laboratory. The separated oil was filtered and then sent to us. It was stored in a full bottle in a refrigerator for a long period before we had an opportunity to examine it, but had remained fresh and free from oxidation. The oil was pale yellow in colour, had saponification equivalent 296·2 and iodine value 146·8, and contained 5·7% of free fatty acid (as oleic) and 0·75% of unsaponifiable matter.

Its component acids were determined by fractional distillation in a vacuum of their methyl esters through an electrically-heated packed column. Before conversion to esters the mixed acids from 205 g. of the oil were first resolved into three groups by crystallization from acetone and from ether at low temperature. It has been shown recently (Hilditch & Pathak, 1947) that low temperature crystallization from solvents affords much more rapid and efficient preliminary separation of the mixed acids of marine animal oils than the procedures hitherto employed, in which use was made successively of the different solubility of their lithium salts in acetone and of their lead salts in ethanol (Hilditch & Maddison, 1942; Bjarnason & Meara, 1944).

In the present instance the herring visceral oil mixed fatty acids (190 g.), after removal of the bulk of the unsaponifiable matter present, were first crystallized from acetone (1900 ml.) at −60° for 5 hr., when 66·9 g. (iodine value 277·0) remained in solution; recrystallization of the deposited acids under the same conditions left a further 17·7 g. (iodine value 236·1) in solution. These two soluble portions were united as fraction *C* (84·6 g., iodine value 268·4). The portion (105 g.) which had remained insoluble in acetone at −60° was then crystallized from ether (1050 ml.) at −30° for 5 hr. when 53·8 g. (*B*, iodine value 89·8) remained in solution and 51·2 g. (*A*, iodine value 23·9) deposited. Nearly all the polyethenoid constituents of the acids were thus obtained in the most soluble portion *C*, whilst the low iodine value of the least soluble portion *A* indicated that only monoethenoid acids were present therein:

Fraction		(g.)	(%)	Iodine value
A	Insoluble in ether at −30°	51·2	27·0	23·9
B	Soluble in ether at −30°	53·8	28·4	89·8
C	Soluble in acetone at −60°	84·6	44·6	268·4

Each group of acids was separately converted into methyl esters, which were fractionally distilled as described above, the most unsaturated group *C* being dealt with first, fractionation and analysis of the ester fractions being carried out as rapidly as possible to minimize chances of autoxidation and polymerization. From the iodine values and equivalents of the ester fractions the composition of the latter was calculated by methods which have been described elsewhere (Hilditch, 1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturation, e.g. −2·0 (monoethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each of the groups *B* and *C*, from which the mean equivalent of each of the homologous ester groups (C_{16}, C_{18}, C_{20} and C_{22}) followed (the values employed are listed in Table 2).

RESULTS

The ester-fractionation data obtained for each of the three groups of herring visceral oil fatty acids are summarized in Table 1.

Table 1. *Fractionation of methyl esters of herring visceral oil acids A, B and C*

Fraction	(g.)	Saponification equivalent	Iodine value
	Methyl esters of acids *A*		
*A*1	2·50	246·0	0·8
*A*2	2·64	250·8	0·9
*A*3	3·68	263·6	0·9
*A*4	3·89	268·0	1·0
*A*5	4·72	268·3	1·2
*A*6	5·41	268·5	1·3
*A*7	4·44	268·9	1·5
*A*8	4·23	270·5	3·0
*A*9	4·30	292·0	29·1
*A*10	3·74	301·6	58·3
*A*11	4·45	302·1	71·3
*A*12	4·95	310·9*	68·7
	48·95		
	Methyl esters of acids *B*		
*B*1	3·34	247·8	5·4
*B*2	3·59	252·8	31·4
*B*3	4·56	281·3	73·1
*B*4	4·65	292·3	87·9
*B*5	4·72	293·3	88·9
*B*6	5·10	297·7	93·8
*B*7	4·83	299·1	102·3
*B*8	4·94	301·3	107·0
*B*9	5·13	302·1	113·7
*B*10	4·36	303·5	118·2
*B*11	2·25	305·9	115·4
*B*12	3·28	320·9*	112·2
	50·75		
	Methyl esters of acids *C*		
*C*1	3·40	250·5	85·9
*C*2	3·94	266·4	123·8
*C*3	3·97	271·4	128·8
*C*4	3·82	277·8	134·4
*C*5	4·04	290·1	156·7
*C*6	5·44	301·8	158·2
*C*7	5·38	307·5	212·2
*C*8	6·24	313·5	318·4
*C*9	5·20	319·4	346·4
*C*10	4·86	327·3	362·9
*C*11	4·03	332·4	377·4
*C*12	5·31	335·9	371·8
*C*13	4·47	351·4*	308·7
	60·10		

* Equivalents of esters (freed from unsaponifiable matter): *A*12, 307·4; *B*12, 314·8; *C*13, 340·8.

Table 2. *Equivalents, iodine values and mean unsaturation employed in calculating the composition of unsaturated methyl-ester fractions from groups B and C*

	B			*C*		
Methyl esters of acids	Saponification equivalent	Iodine value	Mean unsaturation	Saponification equivalent	Iodine value	Mean unsaturation
C_{14}	240·0	105·8	−2·0	240·0	105·8	−2·0
C_{16}	268·0	94·8	−2·0	267·4	123·5	−2·6
C_{18}	295·9	90·1	−2·1	294·3	159·7	−3·7
C_{20}	321·3	185·8	−4·7	317·6	335·0	−8·4
C_{22}	—	—	—	343·5	388·3	−10·5

The percentage composition (by weight) of each group of acids *A*, *B*, *C* was then calculated (Table 3) and therefrom that of the total fatty acids of the herring intestinal oil can be obtained (Table 4).

Table 3. *Composition (% w/w) of the acids in groups A, B and C*

Acid	*A* (%)	*B* (%)	*C* (%)
Myristic	11·2	7·6	1·4
Palmitic	53·1	4·6	—
Stearic	9·2	1·2	—
Arachidic	1·2	—	—
Unsaturated C_{14}	Trace	1·4	2·3
Unsaturated C_{16}	1·2	6·5	18·6
Unsaturated C_{18}	17·5	62·3	20·8
Unsaturated C_{20}	6·5	16·3	35·9
Unsaturated C_{22}	—	—	20·8
Unsaponifiable	0·1	0·1	0·2

Table 4. *Component acids of herring visceral oil*

					Fatty acids	
Acid	*A* (27·0%)	*B* (28·4%)	*C* (44·6%)	Total	(%, w/w)	(%, by mol.)
Myristic	3·0	2·2	0·6	5·8	5·8	7·1
Palmitic	14·4	1·3	—	15·7	15·7	17·0
Stearic	2·5	0·3	—	2·8	2·8	2·7
Arachidic	0·3	—	—	0·3	0·3	0·3
Unsaturated C_{14}	—	0·4	1·0	1·4 (−2·0)	1·4	1·7
Unsaturated C_{16}	0·3	1·9	8·3	10·5 (−2·5)	10·5	11·5
Unsaturated C_{18}	4·7	17·7	9·3	31·7 (−2·6)	31·8	31·4
Unsaturated C_{20}	1·8	4·6	16·0	22·4 (−7·1)	22·4	20·4
Unsaturated C_{22}	—	—	9·3	9·3 (−10·5)	9·3	7·9
Unsaponifiable	Trace	Trace	0·1	0·1	—	—

DISCUSSION

The composition of the herring visceral oil shows marked differences both from that of body (flesh) fats of North Sea herrings (Lovern, 1938) and of Iceland herrings (Bjarnason & Meara, 1944), and from that of the fat in one of the main foods of the herring, the copepod *Calanus finmarchicus* (Lovern, 1935). These differences are illustrated in Table 5.

Mr W. C. Smith, of Port Erin Marine Biological Station, has informed us that *Calanus* is an important item in the diet of herring in Manx waters, and also reminded us that of course the fish do not feed actively at or near the spawning season (early autumn).

The circumstance that the specimen of visceral fat studied probably contained an uncertain amount of herring milt and roe introduces a possibly complicating factor, since Lovern (private communication to the authors) has observed that herring roe glycerides have iodine values of 170 and somewhat higher, and thus may contain large proportions of C_{20} and C_{22} acids of high unsaturation (Lovern, unfortunately, was unable to undertake full analyses of the roe component acids). Since, however, Manx herring at the time of year concerned often include a fair proportion of immature fish, it is on the whole likely that the proportion of milt and roe fat in the total visceral fat which we have examined may not have been very substantial, and that the component acid data in Table 5 represent for the most part those for the fats of the herring viscera.

The chief differences between these visceral fatty acids and those of the herring body fats are in the much lower content of unsaturated C_{22} acids in the former, coupled with much higher mean unsaturation of the acids of both the C_{22} and C_{20} series as compared with those of the body fats; further, the pro-

portion of unsaturated C_{18} acids is much larger in the visceral than in the body fats, although their mean unsaturation is considerably lower. Subordinate differences may include a somewhat lower content of unsaturated C_{20} acids, and somewhat higher contents of total saturated acids and, perhaps, of unsaturated C_{16} acids as well as of palmitic acid.

limits in each case, whilst the only significant differences in proportions of acids present are a larger amount of unsaturated C_{18} and a smaller amount of unsaturated C_{22} acids.

On the other hand, although the mean unsaturation of the various groups (except in the C_{18} acids) is much the same in herring visceral fat and in the

Table 5. *Component acids (%, w/w) of herring body and visceral fats and of copepod fat*

	Body (flesh) fats			Visceral fat Isle of Man (August)	Copepod fat (*Calanus finmarchicus*)
	North Sea				
	July	October	Iceland		
Iodine value	152·5	138·6	140·0	146·8	177·6
Component acids:	(%)	(%)	(%)	(%)	(%)
Myristic	8·3	7·3	7·1	5·8	8·3
Palmitic	12·1	13·0	11·7	15·7	10·6
Stearic	0·3	Trace	0·8	2·8	1·3
Arachidic	—	—	0·1	0·3	—
Unsaturated C_{14}	0·5	0·8	1·2	1·4	1·6
Unsaturated C_{16}	6·4	4·9	11·8	10·5	11·8
Unsaturated C_{18}	21·0	20·7	19·6	31·8	16·8
Unsaturated C_{20}	28·3	30·1	25·9	22·4	24·5
Unsaturated C_{22}	23·1	23·2	21·7	9·3	25·1
Mean unsaturation of:					
Unsaturated C_{14}	−2·0	−2·0	−2·0	− 2·0	−2·0
Unsaturated C_{16}	−3·4	−2·7	−2·4	− 2·5	−2·4
Unsaturated C_{18}	−4·5	−4·2	−3·5	− 2·6	−5·1
Unsaturated C_{20}	−5·5	−4·6	−5·2	− 7·1	−7·8
Unsaturated C_{22}	−4·6	−4·3	−4·3	−10·5	−8·1

Lovern (1938) has suggested that characteristics of herring body fats include an unusually high content of unsaturated C_{22} acids, unusually low unsaturation in the C_{20} and C_{22} acids, and unusually high unsaturation in the C_{16} and C_{18} acids, with lower contents than usual of unsaturated C_{16} acids (although the amounts of total C_{16} acids fall within the usual range). These conclusions were based on his previous statistical survey of the average composition of marine fish fats (Lovern, 1937), in which, however, the data referred to many more liver fats than body fats from marine fish. The statistical data indicated the following approximate amounts and unsaturation of unsaturated acids as being characteristic for marine fish in general:

	(%)	Mean unsaturation
Unsaturated C_{16}	10	−2·0 to −2·5
Unsaturated C_{18}	25	−2·6 to −3·3
Unsaturated C_{20}	25	−5·5 to −6·5
Unsaturated C_{22}	15	−7·0 to −9·5

Turning to the herring visceral fat now under discussion, it will be seen that its component acids align themselves much more closely, both in their proportions and in their mean unsaturation, with Lovern's 'average values' for marine fish fats than with the observed values for herring flesh (depot) fats. The mean unsaturation for the various groups falls within or approximates to Lovern's average

dietary *Calanus* fat, the respective differences in content of unsaturated C_{18} and unsaturated C_{22} acids are as well marked, or even more so, than those between the same groups of acids in the herring visceral and body fats. Lovern (1938) has put forward the view that the herring body fat, with component acids in much the same proportions as in *Calanus* fat, has been stored as a result of ingestion in quantity in the herring diet of the latter fat, but that prior to laying down in the herring fat depots the *Calanus* fat has been modified by considerable hydrogenation of its unsaturated C_{20} and C_{22} components and some dehydrogenation of its unsaturated C_{16} components.

At first sight the observed composition of the herring visceral fat would seem to be at variance with Lovern's explanation, but in our opinion this does not necessarily follow. The visceral fat may be considered, in the herring, to be more of the nature of an organ fat than a depot fat; indeed, its similarity in composition to the 'average marine fish fats' (which as already pointed out were mainly liver fats) may be due to this circumstance. (Fish livers, of course, often function simultaneously as depots, but very broadly speaking the main characteristics of the component acids of fish liver fats are similar, whether the liver is rich or poor in fat.) If the visceral fat of the herring is considered to be an organ fat rather than a depot fat, its dissimilarity to

the specific depot fat laid down by the herring is not necessarily surprising. Thus the liver lipids of land animals (pig, sheep, ox) which have been examined in detail are found, either in their glycerides or their phosphatides, to be respectively broadly similar in composition (Hilditch & Shorland, 1937), and to have some features (e.g. presence of unsaturated C_{16}, C_{20} and C_{22} acids) which are not only common to all these animal liver lipids but are also reminiscent of the glyceride fats of aquatic organisms: but neither the glyceride nor the phosphatide fractions of these liver lipids have any resemblance to the depot fats laid down by the different animals, the depot fats of which moreover differ considerably in composition from one species to another.

We therefore confine ourselves to the conclusion that our present results show that herring visceral fat differs materially in composition from the typical fat laid down in the herring fat depots, and that its component acids are very similar in content and in their degree of unsaturation to those of what appear to be typical marine fish liver fats. It is also evident that much more data could still profitably be sought as to the detailed composition of the fatty acids in different organs and depots from a wide range of species of fish.

SUMMARY

1. The component acids of herring visceral fat have been determined by the ester-fractionation procedure.

2. Preliminary resolution of marine animal fatty acids into groups differing in unsaturation, by means of crystallization from suitable solvents at low temperatures, is shown to be preferable to their separation in the form of lithium and lead salts.

3. The herring visceral oil fatty acids differ from those of herring body fats (and of *Calanus* fat, a fat largely ingested by the herring) in their much lower content of unsaturated C_{22} acids and much higher content of unsaturated C_{18} acids, together with much higher degree of average unsaturation of the C_{20} and C_{22} acids and lower average unsaturation of the C_{18} acids.

We wish to express our thanks to Mr J. R. Bruce for affording us the opportunity to study the specimen of herring visceral fat.

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